FACTORS AFFECTING THE SENSITIVITY TO INSULIN OF DIAPHRAGM AND ADIPOSE TISSUE IN OBESE MICE AND THEIR LEAN LITTERMATES

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Plate 1 : From right to left: ♂ lean, ob/ob fed ad libitum and ob/ob-RD mice, 3 months old.

The body weights of the mice were as follows:

lean fed ad lib	=	39.6 gm.
ob/ob fed ad lib.	=	77.0 gm.
ob/ob-RD " " "	=	41.9 gm.

A B S T R A C T

1. The sensitivity and response to insulin *in vitro* on (a) the incorporation of glucose-U-^{14}C into glycogen in the isolated diaphragm muscle, and (b) the oxidation of glucose-1-^{14}C in the epididymal fat pad and in adipocytes, has been studied in obese hyperglycaemic mice fed *ad libitum* (*ob/ob*), in obese mice maintained on a restricted diet (*ob/ob*-RD), and in lean mice, at 2-4 months of age and at 6-8 months of age.

2. In both age groups there was a significant, although reduced, response to insulin in the muscle from obese mice but this was normal in the 6-8 month old group of *ob/ob*-RD mice.

3. The glucose oxidation in the epididymal fat pad of the obese mice fed *ad libitum* was very reduced in both age groups but it was normal in fat pads from fed obese mice kept on a restricted diet. There was no insulin effect on the fat pad of obese mice in either age group. The response and sensitivity to insulin was partially restored in the *ob/ob*-RD mice.

4. There were only small differences in sensitivity between adipocytes prepared from obese and lean epididymal fat pads.

5. These results suggest that a decreased sensitivity to insulin at the cellular level is not the primary defect in the obese-hyperglycaemic syndrome.

6. When expressed per unit weight of tissue, there were fewer and larger adipocytes, with a smaller vascular space, a smaller water content and the same nitrogen content in pads from ob/ob mice compared to lean mice.

7. The plasma concentration of immunoreactive insulin, (IRI), in the fed or fasted state or after chronic restriction of food intake, was higher in obese than in lean mice. In response to glucose, the plasma IRI concentration 60-90 mins. later was higher in fed or fasted obese mice.

8. These results are discussed together with related findings in experimental and genetic obesity.

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ABBREVIATIONS AND EXPLANATION OF TERMS USED IN THE TEXT

Adipocyte	:	is used synonymously with fat cell but is preferred when specifically referring to fat cells prepared by digestion with collagenase.
AIS	:	guinea-pig anti-ox insulin serum.
FFA	:	free fatty acids.
IRI	:	immunoreactive insulin.
LD^{50}	:	the dose lethal for 50% of a test population of animals.
ob/ob	:	the obese mouse reared under ad libitum feeding conditions.
ob/ob-RD	:	the obese mouse reared from weaning on a restricted diet feeding regimen for 5-7 months.
lean-RD	:	a lean mouse on the same feeding regimen.
acute restriction of food intake	:	refers to a short 10-15 day period of severe food restriction of adult, fully developed ob/ob mice until their body weight was the same as that of lean mice.
S.E.M.	:	Standard Error of the Mean.
VMN	:	animals with stereotaxically placed electrolytic lesions in the ventromedial nucleus of the hypothalamus.

CHAPTER I

INTRODUCTION

1. RELEVANCE TO CLINICAL OBESITY AND DIABETES

Studies of experimental obesity in animals can and have made important contributions to our knowledge of obesity in the human. Experimental approaches under controlled genetic and environmental conditions can be made that are impossible to undertake in clinical investigations. Obesity is widely recognised as a condition of clinical significance because of the consistent reports associating it with an excess mortality proportional to the extent of the obesity[1,2]. A loss of the excess weight by a restriction of the caloric intake results in a return to a normal life expectancy[3]. As the majority of people who contract diabetes in middle age are obese[4], there is some clinical relevance to the study of an animal model of recessively-inherited obesity associated with hyperglycaemia, although the information so obtained would only partly contribute to our knowledge of the polygenic nature of human diabetes mellitus[5].

Hereditary obesity in mice - historical

Hereditary obesity in mice has been studied since the beginning of this century[6]. Owing to the increasing interest in inborn errors in metabolism and in animal models of disease many other types of spontaneous obesity and diabetes have been found in mice[7-15] and other animals[16-23] and have been reviewed by Meier[24], Renold[26] and in the Brook Lodge Symposia[25].

2. INTRODUCTORY DESCRIPTION OF THE OBESE-HYPERGLYCAEMIC SYNDROME - IMPORTANCE OF AGE AT WHICH THE SYNDROME IS STUDIED.

The fully penetrant recessively inherited gene[27-29] ob/ob, which originated in the C57BL/6J strain of mice in Bar Harbor[12*] has been the most extensively studied (reviews[30-33a]) and is the one reported on in this thesis. The inheritance of this gene results in the development of the obese-hyperglycaemic syndrome whose signs course in a characteristic chronological order[33a]. The obesity[33-35], evidenced by an increase in the fat:fat free dry weight ratio, is one of the earliest manifestations of the syndrome[29,33a,33b]. Low spontaneous activity[36,37], imperfect homoeothermia[38,39] and hyperphagia[37,40], accompanied by an apparent preference for high-fat diets[40], are characteristics of the adult syndrome which develops over a 4-5 month period when the disordered energy balance[,41,42] of these mice results in their continued gain in weight (entirely attributable to fat[35]) long after the lean mice have stabilised their adult body weight (Fig.1). After about 6 months of age the gross overweight of the ob/ob mice stabilises, indicating the establishment of a new homoeostasis. For this reason the mice used in this study have been investigated at 2-4 months of age during the lipogenic phase and at 6-8 months of age when the body weight is relatively stable. The disadvantage in the selection of animals by age rather than by weight was that it contributed to the increase in biological variation also caused by the use of a strain of mice which was not pure.

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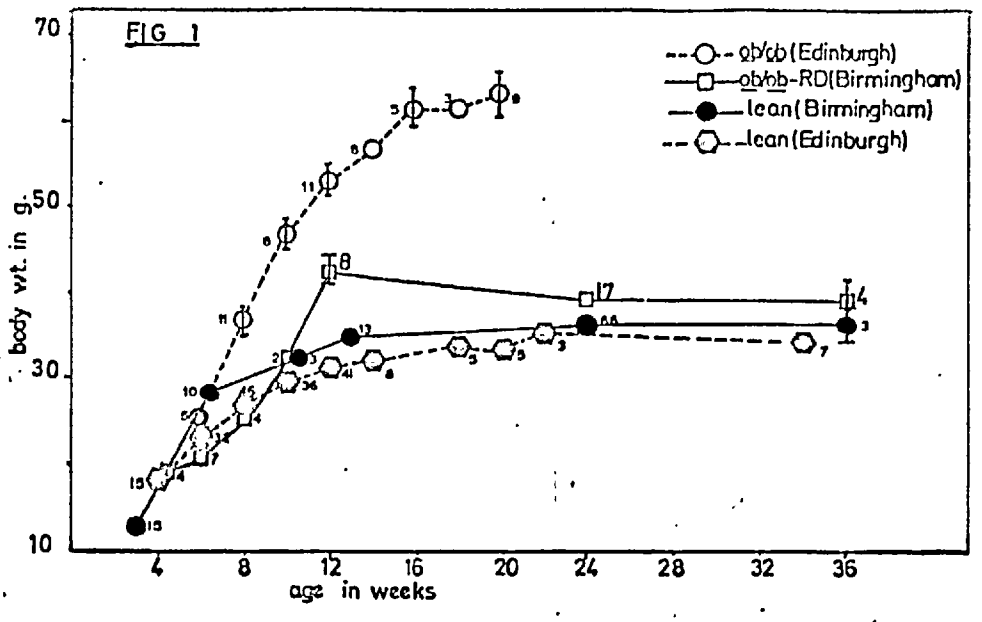
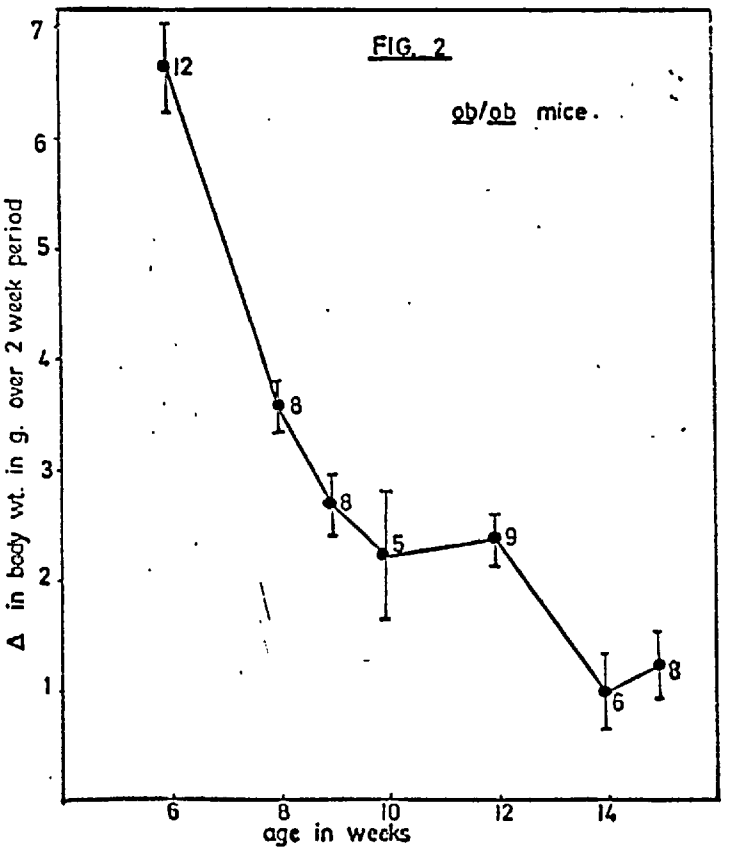


Fig.1 : The body weights of lean and ob/ob mice and of ob/ob-RD mice from the "Edinburgh" and "Birmingham" colonies at different ages.

Fig.2 : The increase in body weight measured over a 2 week period in ob/ob mice of the Birmingham colony at different ages.



3. THE DIABETIC AND HYPERINSULINAEMIC FACETS OF THE SYNDROME

The period of intense fat deposition (with weight gains of 5-6 gm. a week in ob/ob mice compared to 1-1.5 gm. a fortnight / in lean mice (Fig.2)) is characterised by a diabetic syndrome in the fed state including hyperglycaemia and glycosuria (without ketosis)[27-29,33a]. This coincides with the presence of hypertrophy and to a lesser extent, hyperplasia of the islets of Langerhans in the pancreas[27,31,43] with β cells showing histological and chemical signs of hyperactivity[31,44-45], storing and secreting into the blood[27,46-49] paradoxically high levels of an immunoreactive[33a46,48,50] and biologically active insulin[46,48,51]. Further, the plasma insulin of ob/ob mice has a half-life comparable with that of lean mice[52] which implies that the increased destruction of the high circulating levels of plasma insulin in ob/ob mice cannot be invoked to explain the paradoxical glucose intolerance of these mice.

Theories of Insulin Resistance

(i) Peripheral tissue resistance as primary cause

The apparent refractoriness to endogenous plasma insulin is matched by a remarkable tolerance in the fasted state to doses of exogenous insulin in excess of those lethal for lean mice[27-29,33a,53]. After the intraperitoneal injection of as much as 400-600 U/kg. ox insulin into ob/ob mice, severe levels of hypoglycaemia are achieved only after 5-7 hours and are accompanied only infrequently by convulsions. When smaller doses of insulin are injected intraperitoneally or intravenously, only a small fall in their blood sugar results[28,53-55], (although one author finds the absolute fall in blood sugar after 5U/kg.

insulin not to be significantly different in ob/ob and lean mice[33a]).

One of the earliest studies on the action of exogenous insulin on ob/ob tissues in vitro was on the glucose utilisation of the epididymal fat pad where it was shown that in a medium containing 0.36% glucose, although glucose metabolism was considerably depressed in the pads from ob/ob mice, they responded normally to insulin concentrations in the medium of as low as 10^{-4} U/ml.[56]. At the same time, reports of a diminished ability of insulin to inhibit[57] and of epinephrine to augment[57,58] the free fatty acid release of epididymal fat pads in vitro, and a reduced insulin effect on glucose metabolism in vitro,[50,57] attested that a diminished response to hormones in vitro was found concurrent with a reduced glucose metabolism and was related to the ob/ob syndrome since it was absent in obesity caused by the injection of gold thioglucose[50,57,58]. A similar reduction in sensitivity and response to insulin was observed in both diaphragm muscle and epididymal adipose tissue after intraperitoneal injection of insulin[50,59] even at 4-5 weeks of age, a finding which led to the hypothesis that a peripheral insulin resistance affecting muscle more than adipose tissue would lead to a redistribution in the metabolism of blood glucose resulting in increased fat synthesis in liver and adipose tissue[48,59], as has been suggested for human obesity[60-62]. Despite the frequent and common use of the isolated diaphragm muscle preparation incubated in vitro, it had not yet been applied to the study of the insulin resistance of ob/ob mice at the time the work on this study was initiated. The normal rates of incorporation of glucose into glycogen in the isolated diaphragm muscle from ob/ob

mice incubated *in vitro*[63] supported the view then that muscle *per se* did not contribute to the hyperglycaemia and obesity of *ob/ob* mice.

It has been well established that the rodent pancreas possesses a remarkable ability to compensate rapidly to changing functional demands for insulin[64]. An increased demand for insulin induced both by peripheral tissue resistance to the hormone and the resulting hyperglycaemia would therefore result in a hyperinsulinaemic state which, with the hyperglycaemia, would further encourage fat deposition in adipose tissue, in view of the effects of insulin in the stimulation of lipogenesis and possible inhibition of lipolysis in this tissue[65]. This hypothesis that the pancreas of the *ob/ob* mouse has adapted secondarily to the increased functional demands caused either by persistent hyperglycaemia or by tissue insulin resistance leading to hyperglycaemia,[31] gains support from the late development of the hyperplastic, hypertrophic histology of the gland, its histochemical behaviour and its high insulin content, as well as from the largely degranulated appearance of the β cells[47,49] although pancreatic insulin levels are high; also, hyperinsulinaemia is present at an age before pancreatic insulin levels rise above normal[46].

(ii) Hyperinsulinaemia of obesity causing peripheral tissue resistance

Hyperinsulinism is known to occur in a number of clinical syndromes and can be commonly associated with the deposition of fat[62,66-76]. These two associated signs, hyperinsulinism and obesity, would lead one to expect that adipose tissue in hyperinsulinaemic states responded normally to insulin; this has been shown not to be invariably true, and suggests that

resistance to insulin may be an adaptation of an animal to a hyperglycaemic, hyperinsulinaemic state. This evolutionary device, which would be initiated in order to limit the extension of the resulting obesity, would be expected to appear after the development of signs reflecting the primary disorder of the syndrome. Hyperinsulinism, when it occurs pathologically, is not always associated with hyperglycaemia, either in association with or without glucose intolerance[67,68,70-72ab]. It is therefore conceivable that the endocrine pancreas either responds to stimuli other than glucose (such as gastric distension, food substrates, or hormones released by the presence of food), or, under certain conditions, becomes oversensitive to a food or glucose stimulus.

4. SCOPE OF THIS THESIS AND APPROACHES TO TESTING THEORIES OF INSULIN RESISTANCE

The object of this thesis was to establish the significance and nature of the insulin resistance of ob/ob mice and its relation to the possible primary defect - the direct expression of the mutant gene in these animals. Both the hypotheses described above were investigated in an attempt to ascertain in what way the obesity, hyperglycaemia and hyperinsulinaemia were related to each other. It remained possible, of course, that, as in some forms of hereditary obesity and diabetes occurring spontaneously in animals, obesity or diabetes may occur independently[26] and there may not be any causal relationship between them in these species.

(a) The use of the ob/ob-RD animal :

Rabinowitz and Zierler[62], using an *in vivo* in humans forearm preparation, showed that the sensitivity to insulin of the muscle tissue of obese subjects was normal following reduction

of their body weight. Other workers have also reported an increased tolerance for glucose[77,79] and a reduction of the hyperinsulinaemia in obese subjects after weight loss[78-80]. In vitro work on aspirated human adipose tissue has shown a similar marked response to insulin after weight reduction of obese subjects who previously possessed insulin insensitive adipose tissue[80]. It was, therefore, felt important to make a study of obese mice on a restricted diet (ob/ob-RD) as they are hyperactive and would serve as a control for the hyperphagia, inactivity and sterility[81,82] found in ob/ob mice fed ad libitum. It had been shown earlier that, when reared on a restricted diet of 2.5 g. a day from weaning, such as to maintain a normal body weight, ob/ob mice lost two of the distinguishing characteristics of the obese-hyperglycaemic syndrome of the fed ad libitum state: their tolerance to exogenous insulin and their hyperglycaemia[54]. These mice, who despite their normal body weight, are still obese[83ab], also have a normal life expectancy[84] and are fertile[82]. The inclusion of a group of these mice in most experiments was an important factor in the experimental protocol designed to indicate how dependent the hyperinsulinaemia and tissue insulin sensitivit were on (i) the high blood sugar concentration, which is normal in ob/ob-RD mice and (ii) the hyperphagia and low spontaneous activity of the ob/ob mice fed ad libitum which are absent in the ob/ob-RD mouse.

(b) The in vitro sensitivity and response to insulin of adipose tissue and muscle :

After the discovery that the serum insulin from ob/ob mice was biologically active[46,48,51] and was destroyed at the same rate in vivo as in lean mice[52], it became clear that the resistance to exogenous insulin found in ob/ob mice was

probably due to a defective insulin response system. The actions of insulin *in vivo*, (other than on increased potassium uptake and reduced free fatty acid release in muscle[85]) are primarily concerned with the removal and subsequent metabolism of blood glucose and have been adapted for use as an *in vivo* bioassay[86]. In this study the response and sensitivity to exogenous insulin has been studied *in vitro*, using tissues from fed lean mice, fed and 24 hour fasted *ob/ob* mice fed *ad libitum* and fed *ob/ob*-RD mice at 2-4 months and 6-8 months of age.

Definitions:

It is opportune at this stage to define the terms 'sensitivity' and 'response' to insulin as used in this thesis. A response is a significant difference in the parameters examined with and without insulin, expressed either as an absolute increase in that parameter or as a percentage increase of the basal value of that parameter, (viz. in the absence of insulin). Sensitivity is measured as the minimum concentration of insulin at which a response is significantly detectable, viz. the detection limit of the assay[87]. This latter variable would of course depend on the accuracy at which the basal value was measured in any particular bioassay.

Information to be gained:

Diminished response to insulin occuring only after the body weight of the *ob/ob* mouse had stabilised would indicate that *in vitro* resistance to insulin was a secondary adaptation to the hyperglycaemic hyperinsulinaemic state of the *ob/ob* mice at this age. A normal response and sensitivity to insulin in the lipogenic phase of the obesity between 2-4 months of age

would indicate that the hyperglycaemia and increasing hyperinsulinaemia were contributing to or causing the excessive fat deposition. The same experiments would provide information on the basal metabolism of glucose in isolated tissues from lean and ob/ob mice *in vitro*, and would therefore ascertain whether a reduced peripheral uptake of glucose, occurring at the early lipogenic phase, was also contributing to an increased demand for insulin.

Significance of use of *in vitro* methods :

In vitro methods have a number of obvious advantages over *in vivo* methods. Where animals are in relatively short supply, as in this instance, more than one determination can be obtained from each tissue, (usually a control and one or more variables), and two or more tissues can be investigated from each animal. This enables the construction of insulin dose-response curves from both adipose tissue and diaphragm muscle from the same animals. As other hormones[88,89ab], endogenous bound insulin[89a-91], glucose[92-94], plasma proteins[96,97], pH[98] and sodium[99] are all known to affect the sensitivity and response of peripheral tissues to insulin, and as other proteins found in plasma possess insulin-like activity[95,100-103], *in vitro* methods are advantageous in the control of the experimental situation that they offer. In particular, the use of isolated adipocytes enabled the measurement of dose-response curves with a third preparation and at two different glucose concentrations, under conditions where extra-adipocyte elements, including probably bound hormones[104], were absent.

Significance of choice of tissue

Adipose tissue has been shown to be a major site of carbohydrate and fat metabolism[65], the metabolic activities of which are regulated by numerous hormones and in particular, insulin[65]. It has been regarded as the quantitatively major anatomical site of action of insulin on lipogenesis from glucose, a major metabolic fate for this substrate[105]. The facts that most of the excess body weight of the ob/ob mouse is concentrated in adipose tissue, that ob/ob mice are hyperglycaemic and that adipose tissue is known to be important in the synthesis of long chain fatty acids from glucose[65] were the reasons for studying this tissue in vitro. The epididymal fat pad has been the most extensively studied organ of adipose tissue[65], , partly because it is so discrete a tissue, and partly because its veil-like morphological features minimise diffusion artifacts when it is incubated in vitro.

The rat diaphragm preparation, which was used to show the first in vitro effect of a hormone[106], is a suitable in vitro preparation for much the same reasons. It was developed for the study of muscle metabolism[107] and as a bioassay for insulin in rats[108-111] and mice[112-115] after the original use of glucose-U-^{14}C to study the incorporation of glucose into glycogen and other metabolites[116].

Ideally suited though they may be to in vitro incubation work, the isolated diaphragm and epididymal fat pad cannot be assumed to be quantitatively representative of the total muscle and adipose tissue mass of the animal[117], although qualitatively they have been reported as being representative of skeletal muscle[118] and white adipose tissue[117,119] respectively

in their metabolism of glucose and response to insulin. Epididymal adipose tissue in particular is known to possess remarkable "individuality" in its rate of enlargement under different conditions[120]. However, both Christophe[56,121], who studied mesenteric adipose tissue, and Chlouverakis[55], who studied omental adipose tissue and abdominal muscle from lean and ob/ob mice, obtained results that were qualitatively the same as those obtained with epididymal fat pad and hemidiaphragm tissue respectively.

In vitro bioassays for insulin and choice of parameters to be measured - (i) diaphragm muscle :

Of the many biological actions of insulin on hemidiaphragm muscle in vitro, the effect on glucose uptake and the incorporation of glucose-U-^{14}C to glycogen is most relevant to this study. The rat diaphragm bioassay for insulin, one of the earliest in vitro bioassays for insulin is based on the effect of insulin in increasing glucose utilisation, particularly glycogen synthesis, by rat and mouse diaphragm. Wardlaw[114], in his studies with mouse hemidiaphragms, showed that the percentage error in the measurement of increase in incorporation into glycogen was much less than that of glucose decrease in the medium. Accordingly, incorporation of glucose-U-^{14}C into glycogen was used as the indicator of insulin action on diaphragm muscle in these studies. Quarterdiaphragms were used in preference to hemidiaphragms as, although increasing the scatter of the observations[114], their use did not affect the response and sensitivity to insulin[114] and facilitated the construction of dose-response curves.

(ii) epididymal fat pad :

Many of the actions of insulin on intermediary metabolism in adipose tissue have been shown in vitro[65] and some have been used as the basis for bioassays of the hormone[103,122-127]. In particular, for adipose tissue, the studies of Winegrad[105,128], Martin[125] and Renold[126,127] and their co-workers were used as models on which the present assays were based. One of the most sensitive parameters for insulin bioassay is the stimulation of the oxidation of glucose-1-^{14}C to $^{14}CO_2$ by the hexosemonophosphate pathway[103,125-129]. Although this parameter is not specific for serum insulin assayed on the epididymal fat pad[65,100,124,127], it is highly specific for small quantities of insulin (10^{-5}-10^{-7}U/ml.) when assayed with isolated adipocytes; in either case, serum factors would only influence the assay by virtue of tissue adsorption persisting after the preincubation washing with buffer. The use of glucose-1-^{14}C oxidation as a parameter of insulin action affords further advantages in that it is relatively easy, quick and accurate to perform and is economical in its requirements for radioactive substrate, (or alternatively the amount of tissue used). It has been the parameter of choice in the work on adipose tissue sensitivity and response reported here.

Studies on adipose tissue cellularity :

The fact that nearly all of the excess weight of the ob/ob mouse and of its adipose tissue is composed of fat[34,130] without any proportional increase in adipose tissue water, body water[131] or fat-free dry weight[130] casts doubt on the validity of metabolic data expressed per unit $\frac{\text{wet}}{\text{weight}}$ or per unit fat content

in the absence of data on the number of metabolically active cells involved. Further, the histological appearance of adipose tissue from ob/ob mice has been shown to be very different from that found in lean mice[132-134] and confirms the very obvious macroscopic differences in size, texture, colour[56] and fragility that can be observed in this tissue. Numerous non-cellular fat cysts have been found as well as a variety of cells from the reticulo-endothelial system[132], and, in particular, mast cells, occurring in numbers 50 times greater than found in lean mouse adipose tissue[135].

(iii) The isolated adipocytes preparation

These particular observations made it imperative to study the adipose tissue of ob/ob mice in a manner which obviated these differences and which facilitated the study of glucose metabolism and insulin sensitivity and response of the adipose cell uncomplicated by the presence of other cell types and stromal or vascular elements. The method of preparing isolated adipocytes, first described by Rodbell in 1964[136] for tissue from rats, has been particularly useful in this instance and has been modified for use with epididymal adipose tissue from lean and ob/ob mice. Apart from the removal of non-adipocyte elements, this preparation has the advantage that it has a high index of precision, is extremely sensitive to insulin[136] and is extremely economical of tissue, a factor of importance in studies on mice, (despite the higher percentage contribution to the total body weight of the epididymal fat pads in these animals[56]). Insulin dose-response curves using adipocytes and medium glucose concentrations of 0.03%, 0.1% and 0.2% could be compared with

the results obtained using intact pieces of epididymal fat pad from lean and ob/ob mice in order to assess the contribution of extra-adipocyte elements to the sensitivity and response of adipose tissue to insulin.

(c) In vivo plasma insulin and blood glucose concentrations :

Because of the close association suspected between tissue insulin resistance and plasma insulin concentration and because measurements of these two parameters differ widely in different laboratories, it was felt necessary to supplement the present study with data on plasma immunoreactive insulin and blood glucose concentrations and the influence of fasting and glucose on these concentrations. These experiments were done in collaboration with E. Dade, J. Elliott & Dr.D.A.Hems in a combined study on liver glycogen synthesis and adipose tissue free fatty acid mobilisation in ob/ob, ob/ob-RD and lean mice in vivo.[324] Accordingly, plasma insulin and blood glucose concentrations were measured in the fed and fasted state in lean and ob/ob mice who were fed with various glucose loads intragastrically and subcutaneously. It was hoped that these experiments would indicate how the hyperinsulinaemia of ob/ob mice was related to their hyperglycaemia.

Many factors including some gastrointestinal hormones have been shown to affect insulin secretion[137,138]. It was important to confirm that glucose was a major stimulus of insulin secretion in ob/ob mice. If this was so, one should expect plasma levels to increase in proportion with the extent of the hyperglycaemia. The control of the glucose intake of the mice and the concurrent measurement of plasma insulin and blood glucose levels

served to test the hypothesis that the observed hyperglycaemia, and its consequences, in ob/ob mice in the fed ad libitum state are not simply the result of the fact that, being hyperphagic, they are more often studied in a "fully-fed" state. Alternatively, as in some clinical syndromes, the hyperinsulinaemia may be correlated more closely with the degree of obesity, or with the development of insulin resistance in the peripheral insulin-sensitive tissues, than with the blood glucose concentration.

5. DESCRIPTION OF FORM OF PRESENTATION OF EXPERIMENTS.

The presentation has been divided into Experimental, Results and Discussion of Results. Within each of these three sections, diaphragm experiments come first, followed by epididymal fat pad, isolated adipocytes, ob/ob fat pad morphological characteristics and *in vivo* plasma IRI and blood glucose measurements. The concluding section attempts to place this work in the context of current research into spontaneously occurring genetic obesities in mice, and to suggest promising paths for future research.

CHAPTER II

EXPERIMENTAL

A. ANIMALS

(i) Strain of ob/ob mice.

The unfortunate recurrence on two occasions of infectious disease in our mouse colonies necessitated the maintenance of two colonies of mice. Both colonies originated from the Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A.; heterozygous carriers from that laboratory were introduced into local "mixed" colonies in (i) Edinburgh and (ii) Birmingham, from which our original stocks were obtained. In much of the preliminary work the animals used were bred from the Edinburgh colony, and for nearly all the other experiments animals bred from the Birmingham colony were used. The Edinburgh colony were uniformly buff in colour while the Birmingham colony included albinos as well. During the course of the studies reported here no other phenotypical differences between the two colonies could be detected.

(ii) The Maintenance of the Mice.

The mice were housed in heated rooms at 21-22.5°C (relative humidity 60) in 11" x 8½" x 4¼" transparent polycarbonate cages containing either 1 - 6 ob/ob mice and their lean littermates (used for breeding) or 10 - 15 lean and ob/ob mice in 18" x 13" x 6½" stock cages. The lighting, from two

pairs of 8 watt fluorescent tubes came on at 8 a.m. and switched off at 8 p.m. every day. The obese mice were usually housed with the littermates of their sex except very recently in the *in vivo* work when all the *ob/ob* mice were housed together. The animals were fed *ad libitum* Thompson's Rat and Mouse Research Diet, (supplied by Pilbury's Dietary Animal Foods, Birmingham), whose composition is given in the Appendix (p.216).

(iii) *Obese mice on a restricted diet (ob/ob-RD)*

The *ob/ob* mice which were reared on a restricted diet (and referred to from now on as *ob/ob*-RD mice) were housed individually in cages free of sawdust or peat, (blotting paper was used as bedding). They were separated from their littermates as soon as obesity could be indisputably detected by eye, (approximately between 28 - 35 days of age), and fed the following amounts of milled Thompson's Diet in the early morning:

Age in weeks	Amount of food
4	3.7 g.
5	4.4 g.
6th week onwards	5 - 6 g.

These figures were obtained from a pilot experiment involving the measurement of the free food intake of two lean mice (♂ + ♀) from the Edinburgh colony.[139] Later experiments, with lean mice from the Birmingham colony fed this diet, resulted in slightly smaller, though fit and active mice that were definitely underweight for their age (25 - 30 g. at 6-8 months of age).* The food intake of adult *ob/ob* mice in our colony has now been

* Incomplete consumption of the daily food ration because of soiling with excreta was the main reason for this inadequate nutrition.

measured, and has found to be about 140 g. a day. The continued use of this dietary regimen was justified in that although it may have provided slightly less than lean mouse caloric requirements (which were difficult to establish to within < 10%), ob/ob-RD mice on this regimen attained a body weight only slightly higher than that of lean mice at the same age and showed no obvious effects of ill health or of a negative nitrogen balance. These hyperactive animals always ate their single daily ration in a few hours. For this reason, sampling of tissues was always carried out in the afternoon so that the mice were studied in the postabsorptive state. Lean mice, pair-fed as controls, but not studied here, had a mean body weight that was significantly less than the comparable weight for lean mice fed ad lib. (Table 1) This suggests either that these mice were undernourished or that the feeding regimen could introduce other metabolic and hormonal changes independent of caloric intake.

(iv) Rate of Gain in Weight.

The growth curve in Fig. 1 shows the rate of attainment of overweight in ob/ob mice of the Edinburgh colony; the average body weight of these mice in the different age groups used is given in Table 1. The mean body weights of the mice of the two colonies at any particular age group were very similar. Two other local strains of lean mice were used; a mixed albino strain, designated BSVS (bacteria-sensitive, virus-sensitive), and lean mice of the CBA- strain. Male Sprague-Dawley rats (150-250 g.) were used in a few studies.

TABLE I

Mean Body Weights of lean and obese mice and of obese mice on a restricted diet at different ages.

TYPE OF MOUSE	AGE MONTHS	WEIGHT g.
Lean	2-3	30.3 ± 0.4 (48)
ob/ob	"	62.3 ± 1.2 (22)
ob/ob-RD	"	42.5 ± 1.7 (8)
Lean	5-6	36.2 ± 0.5 (66)
Lean-RD	"	26.5 ± 1.8 (7)
ob/ob	"	78.4 ± 2.3 (36)
ob/ob (Edinburgh)	"	70.2 ± 3.0 (17)
ob/ob-RD	"	38.8 ± 1.5 (17)
Gold thioglucose obese	"	44.8 ± 1.9 (8)
VMN-lesioned mice	"	49.0 ± 3.1 (4)
Lean ♂	6-8	39.6 ± 1.0 (17)
ob/ob ♂	7-9	85.9 ± 3.9 (13)
ob/ob-RD ♂	9	39.3 ± 2.0 (4)
ob/ob ♀	12	79.2 ± 7.8 (6)

Mice were of mixed sex from the Birmingham colony unless otherwise stated; in general, male mice were heavier than female mice. The mean body weights which were usually obtained in the afternoon, are given with the S.E.M.; the number of mice weighed is in parenthesis.

(v) Use of Two Age-Groups.

It is clear that ob/ob mice increase in body weight during a very restricted period from about the first to the fifth month of age. This is more clearly shown in Fig. 2,

where the change in body weight over a fortnight has been plotted against age. As the excess body weight is in the form of fat (see Table 19), this phase has been called the lipogenic phase. It is characterised by slowly increasing plasma IRI levels and by high fed blood sugar levels.[33a] The body weight of obese mice stabilises after five months and this phase, called the static phase of obesity, is one in which the adaptations to the primary cause of the obesity are prominent. In this phase normal [33a] or high [46,48,54] blood sugar levels are evident and the plasma IRI levels reach their maximum soon after the body weight has stabilised.[33a]

Fig.1 also shows some specimen growth curves of obese mice on the restricted diet mentioned above. It can be seen that these follow the body weight curves of the lean mice quite closely and the lipogenic phase was not apparent. Although of greatly reduced body weight, these mice had an appearance that was distinctly obese (see Plate 1), the flanks and rump being much more rounded and puffy than in lean mice. The excess subcutaneous fat that was responsible for this and the much larger quantities of epididymal adipose tissue obtained were reflected in the higher fat/fat-free dry weight ratios that these mice still possessed.[83a,83b]

(vi) Lean Mice made Obese by Stereotaxic Lesioning in the Hypothalamus.

Following the discovery that stereotaxically implanted electrolytic lesions in the ventromedial nucleus of the hypothalamus led to obesity through the development of hyperphagia [141,142], Mayer [143] adapted the technique for use in mice

in order to compare a "regulatory" type of obesity (viz. animals made hyperphagic by brain damage) with a "metabolic" type (e.g. hereditary obesity). In view of Mayer's extensive work supporting this hypothesis of two contrasting aetiologies for obesity [144], an attempt was made to obtain some mice made obese by electrolytic lesions in the ventromedial nucleus of the hypothalamus. Very few of these mice were successfully produced* but two preparations of isolated adipocytes from the epididymal fat pads of these mice were incubated and their sensitivity and response to insulin tested. The rate of increase in body weight of lean mice after the lesioning operatio is shown in Fig.3.

The stereotaxic instrument was made by La Précision Cinématographique, 19, rue des Parisiens, Asnières, Seine, France.

Lesioning Operation

The mouse was anaesthetised with 0.4ml. Avertin (1 g./ml.) and mounted with the horizontal axes of the instrument correctly implanted in the external auditory meatus of the mouse's head. The central axis was adjusted under the maxillary bone and the electrode's zero was set to coincide with its position over the lambda point in the sagittal suture. A small patch of skull hair was removed with a swab of toluene and the skin was incised just under the calculated stereotaxic point of entry of the electrode. A hole was drilled through the skull and meninges at this point and the electrode gently lowered into position and the lesion made with a timed pulse of current. The electrode zero position was re-checked before the wound was dusted with antibiotic and sewn up.

* All lesioning procedures were performed by Dr. R. Batt.

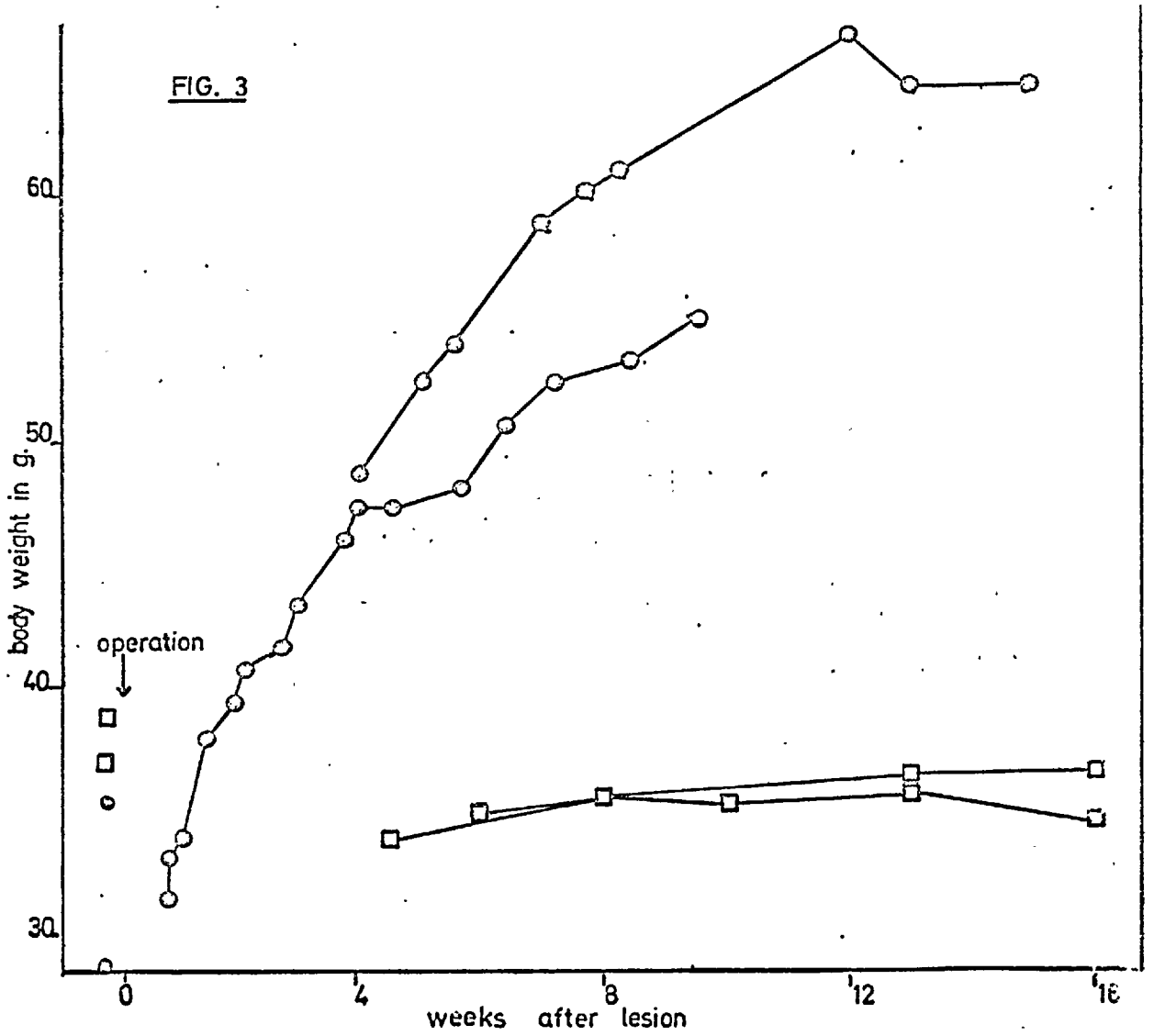


Fig.3 : The increase in body weight in VMN-lesioned lean mice. The closed circles refer to mice that became obese after the lesioning; the open squares refer to similarly treated mice which did not become obese. The operation was usually performed when the mice were 8 -12 weeks old.

Sterile precautions were taken throughout. The brains were fixed in formaldehyde on the death or use of the mice, and the exact position of the lesion determined histologically.

(vii) Induction of Obesity with Gold Thioglucose.

Obesity in mice has been induced by single injections of gold thioglucose [145,146], an agent that has been shown to produce lesions predominantly in the ventromedial nucleus of the hypothalamus [147,150], but also including other extrahypothalamic areas [151]. Obesity in the lean BSVS and CBA strains of mice was induced by the injection of 0.5 - 0.9 mg./g. gold thioglucose (purchased from Sigma Chemical Co., London) intraperitoneally or intravenously in a single 0.5 ml. dose in saline in 8-12 week old male mice. All injections were administered between 11.00 - 12.00 hours.

The age and strain of the mice used [151,152], their metabolic state [146], the amount of concurrent glucose or insulin administered [146,152,153], the time of day [154] and the ambient temperature [148] at which the injections were given and their route of administration have been shown to influence the LD^{50} dose of this agent.

A number of protocols, varying dose with concurrent glucose administration, were tested in order to ascertain the optimal dose and method of administration for the particular species of mouse used. These are shown in Table 2 overleaf. Body composition studies were done only on the mice that became obese (body weight $>$ 40 g.).

In agreement with other studies, it can be seen in Table 2 that glucose, by acting competitively with the gold thioglucose, had some protective action when administered

TABLE 2 : Incidence of Obesity and Mortality in Lean Mice Injected with Gold Thioglucose

STRAIN OF MOUSE	NO. OF MICE	AGE WKS.	DOSE mg/g body weight	TEMP.	ADDITIONS	METHOD OF ADMINISTRATION	% MICE >40g.	% MORTALITY UP TO ONE WEEK AFTER TREATMENT
BSVS ♂	10	8-12	0.8	22°C	25 mg.glucose	Intraperitoneal	0%	40%
"	10	8-12	0.5	22°C	50 mg.glucose	"	0%	10%
"	10	8-12	0.5	22°C	100 mg.glucose	"	0%	10%
"	10	8-12	0.8	22°C	50 mg.glucose	"	20%	40%
"	8	8-12	0.8	22°C	100 mg.glucose	"	0%	25%
"	9	8-12	0.5	22°C	50 mg.glucose	Intravenous	11%	33%
CBA ♂	10	11	0.8	23.5°C	100 mg.glucose	Intraperitoneal	80%	20%
"	10	"	0.6	"	100 mg.glucose	"	60%	0%
"	10	"	1.0	22.5°C	100 mg.glucose	"	0%	100%
"	9	"	1.2	"	100 mg.glucose	"	0%	100%
"	9	"	0.9	26°C	100 mg.glucose	"	11%	-
"	7	12	0.8	18.5°C	-	"	14%	-
"	8	12	0.8	"	-	"	50%	-

Total injection volume was always 0.5 ml. saline.

simultaneously with gold thioglucose which is why the time of day at which injections are administered is so important. Also, if the gold thioglucose was given intravenously, a smaller dose was needed to induce obesity. The optimum dose for a particular strain of mouse, (note the more sensitive response in CBA mice, also seen by Liebelt [149], compared to BSVS mice), was small enough to reduce mortality but large enough to be effective in inducing obesity.

B. MATERIALS

(i) General

All chemicals were of analytical grade unless otherwise stated.

Oyster glycogen, mineral salts and glucose of microanalytical grade were obtained from B.D.H., Poole, Dorset.

Crystalline ox pancreatic insulin (23.4 U/mg.) and insulin binding reagent were obtained from Burroughs Wellcome and Co., Beckenham, Kent.

Crystalline bovine albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Collagenase Type III b, chromatographically pure with no proteolytic activity was obtained from Sigma Chemical Co., London or from Worthington Biochemical Corp., (Code CLSPA).

Lithium heparin from Evans Medical Ltd., Speke, Liverpool.

Ethanol from James Burrough Ltd., London, S.E.11.

95% O_2:5% CO_2 and 100% O_2, for medical use, were obtained from the British Oxygen Co.

For glucose estimation :

Horseradish peroxidase RZ 1.0 from Hughes and Hughes.

O-tolidine

Fermcozyme 653A (750 U/ml) from Hughes and Hughes.

Glacial Acetic acid, sodium iodoacetate, sulphate and acetate of A.R. grade from B.D.H., Poole, Dorset.

For gastric intubations :

15 cm. "Portex" polythene cannulae from Portland Plastics Ltd., Hythe, Kent.

For cell counting and sizing :

OsO_4 and collidine from B.D.H., Poole, Dorset.

(ii) Radioactive materials

All radioactive materials and the oxoid membrane discs for the insulin radioimmunoassay were purchased from the Radiochemical Centre, Amersham, Bucks.

(i) (U-^{14}C) glucose :sp.act. 2-4 mC_i/mM

(ii) (1-^{14}C) glucose : type CFA 356 of low sp.act. 261 μC_i/mM

The (1-^{14}C) glucose of low specific activity was used as high

yields of $^{14}CO_2$ were obtained from the type of high specific activity on the simple addition of 2NHCl in the absence of tissue or of bacterial contamination.

(iii) ^{125} I-human serum albumin : IMI7P 50μC_i/ml. 0.5% albumin

(iv) ^{125} I-Insulin : IM38 5μC_i/5ml; 0.1μg insulin in phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin and 0.025% thiomersal

(v) (^{3}H-1)-Sorbitol : TRA 288 200 mC_i/mM

(vi) $Na_2{}^{14}CO_3$ in aqueous solution : CFA 2 55.0 mC_i/mM or 1mC/0.5 ml.

C. METHODS

IN VITRO EXPERIMENTS

GENERAL

(i) Composition of Buffer Solutions :

The Krebs-Ringer buffers [155] used with diaphragm and adipose tissue were made up as shown, except that the suggested amount of $CaCl_2$ has been halved as recommended by Rodbell [136] for use with isolated adipocyte suspensions. Experiments using hemidiaphragm muscle were performed with double the quantity of Ca^{++} used in all the other in vitro incubations of quarter-diaphragms, epididymal fat pads and adipocyte suspensions.

TABLE 3

SALT	MOLARITY OF STOCK SOLUTION	PARTS BY VOLUME	
		Phosphate Buffer	Bicarbonate Buffer
NaCl	0.154	100	100
KCl	0.154	4	4
aq.$CaCl_2$(70-75%)	0.110	3	3
KH_2PO_4	0.154	1	1
$MgSO_4.7H_2O$	0.154	1	1
$Na_2HPO_4.12H_2O$	0.100	20	-
$NaHCO_3$	0.154	-	21

The Krebs-Ringer bicarbonate was gassed with 95%O_2:5%CO_2 before use, after which it reached a pH of 7.4.

The Krebs-Ringer phosphate buffer was adjusted to pH 7.4 with NHCl.

Buffer for Incubation of Liver Slices : A bicarbonate medium[156] with the following composition was used for the incubation of mouse liver slices.

$KHCO_3$	0.04M	444 mg.	dissolved in 100 ml. of H_2O ; pH 7.4
KCl	0.07M	638 mg.	
$MgCl_2 6H_2O$	0.02M	451 mg.	
$CaCl_2 2H_2O$	0.01M	163 mg.	

All distilled water for buffer solutions was deionised prior to use.

(ii) Insulin; Preparation of Standards :

The crystalline insulin was stored dry at 5°C. 1-3 mg. insulin were dissolved in deionised water by the addition of the minimum quantity of 0.1 NHCl necessary to clarify the solution. For in vitro incubation experiments, dilutions were prepared daily from a stock solution (5-10U/ml), (solutions were always discarded if turbid to any degree), which was prepared weekly, stored at 5°C and opened only after equilibration at room temperature. As insulin adsorbs to glass[157], all insulin solutions contained 0.2% dialysed crystalline bovine plasma albumin and were stored in glass vials.

Standards for the immunoreactive assay of insulin were prepared as 100µl. aliquots of a 200µg/ml. solution stored at -18°C and used over a period of 18 months. Fresh standards prepared at six monthly intervals showed that no significant deterioration of the original standards had occurred.

(iii) Albumin :

The crystalline bovine albumin was stored dry at 5°C. Human albumin has been shown to possess insulin-like biological activity in adipose tissue [101,103,160,161] and muscle [95,158] In fact, immunoreactive insulin [158,159] and non-suppressible insulin-like activity [95,103,160,161] have been found in human serum albumin. A number of batches tested after dialysis on isolated adipocytes in vitro, possessed minute amounts of insulin-like activity. The batch with the smallest biological activity, batch MK2970, was used throughout. The albumin used (as a 30% solution in 300% hypertonic buffer) was always dialysed overnight for 12-18 hours at 5°C against 2-3 litres of the same buffer before use the next day.

TISSUE PREPARATIONS IN VITRO

Diaphragm Muscle

(iv) Removal of Diaphragm Muscle :

After cervical dislocation, the abdomen of the mouse was opened and the liver pulled down away from the diaphragm. The diaphragm was then dissected and removed from the body cavity as described by Wardlaw[114] and placed immediately in saline cooled in an ice bath. Holding the diaphragm by its central tendinous area, it was shaken gently in the saline to wash it free of blood. After a short 1-3 minute period of soaking[112], (soaking has been shown not to influence the insulin effect[114]), during which time other organs were excised from the mouse, it was placed on filter paper and dissected so as to exclude the thick central posterior muscle attached to the arcuate tendon which is separated from the main crescentic area of diaphragm muscle by connective tissue. All bone and connective tissue were also dissected off the muscle which was then cut into two (20-30 mg. each) or four (10-15 mg. each) equal pieces. The dissection of the diaphragm was achieved in two stages by blotting and dissecting each hemi- or quarterdiaphragm at a time so as to minimise the time the tissue was not in buffer. Each piece was weighed on a torsion balance and placed in a vial containing incubation medium, glucose-U-^{14}C, and either water or insulin in 0.2% albumin solution.

(v) The Incubation Medium for Diaphragm Muscle :

1.6 ml. of Krebs-Ringer bicarbonate or phosphate buffer (125% hypertonic solution) with 0.2% dialysed crystalline bovine albumin *. Sufficient glucose was added to achieve the desired final concentration of about 0.1% glucose.

0.2 ml. 0.2% dialysed crystalline bovine albumin or insulin of the required concentration in 0.2% albumin.

0.2 ml. Glucose-U-^{14}C ($\simeq$ 1-5μC_i) in water.

2.0 ml. Total volume

(vi) Incubation Procedure for Diaphragm Muscle :

In all experiments with quarterdiaphragms, one part of the diaphragm was incubated without insulin and the other three parts with different concentrations of insulin; when hemidiaphragm were used, one was incubated without, and one with, insulin. The concentrations of insulin studied are indicated in the results. Incubations were carried out in Warburg vessels or glass vials (Packard) containing 2 ml. of Krebs-Ringer phosphate or bicarbonate buffer. The bicarbonate buffer was always gassed with 95% O_2: 5% CO_2 for at least 10 minutes prior to receiving the diaphragm muscle, after which the vial was closed and incubated at 37°C for 90 minutes in a metabolic shaker: (maximum divergence of insulin and control values occurs at 90 minutes[114]).

(vii) Measurement of O_2 Consumption of Mouse Hemidiaphragm :

These incubations were carried out in Warburg vessels with attached manometers. The CO_2 produced was absorbed by 0.15ml

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* Footnote: Albumin was not present in the medium in experiments involving the chromatography of diaphragm tissue extracts.

Hyamine (hydroxide of Hyamine 10-X, IM solution in methanol, from Packard Instrument Co. Inc., Illinois 60515, U.S.A.), placed in the centre well with a small piece of fluted filter paper to increase the area available for absorption. After placing a hemidiaphragm in each Warburg vessel kept on ice, the tissues and surrounding buffer were gassed with O_2 for 10 minutes and then equilibrated in the water bath at 37°C for a further 10 minutes before any manometer readings were recorded. After adjusting the manometers to an arbitrary "zero" on the scale, readings of the change in pressure were taken every 10 minutes.

Liver

(viii) The Incubation Medium for Liver Slices :

1.6 ml. Bicarbonate medium (125% hypertonic) with no added albumin, and glucose (allowing for the added glucose-U-^{14}C) to a final concentration of 0.2%.

0.2 ml. Deionised water or insulin solution, (no albumin).

0.2 ml. Glucose-U-^{14}C in water (1-5μc).

2.0 ml. Total volume

(ix) Incubation Procedure for Liver Slices :

After removal from the animal, the liver was washed in ice-cool saline and suitably sized pieces (60-220 mg. each) were cut and then placed in a Stadie-Riggs microtome[162]. Slices were cut as thinly as the consistency of the liver would allow and were blotted, weighed and transferred as rapidly as possible to glass vials containing bicarbonate medium, (total volume 2 ml., with a final glucose concentration of 0.2% and 5μC_i of glucose-U-

One slice was placed in each vial, which was gassed with 95% O_2:5% CO_2 before incubation for one hour at 37°C with slow shaking (78 C.P.M.). Unfortunately during many of the incubations

the liver slice disintegrated in the medium, making quantitative retrieval of the tissue very difficult. This was especially true of slices from ob/ob mice where the fatty infiltration of the liver made this preparation particularly unsuitable. Tissue that was retrieved from the vials at the end of the incubation was washed in three changes of saline and treated as described for diaphragm muscle in section (xii).

(x) Preparation of Tissue Samples after Incubation :

At the end of 90 minutes, the vials (or Warburg vessels) were removed from the water bath and placed in an ice bath in a fume chamber. The pieces of diaphragm were individually removed from the incubation mediums, blotted and washed three times using iced normal saline with blotting on filter paper between each wash. They were then either placed directly into a test-tube containing 0.4 ml. 30% KOH and 10 mg. oyster glycogen, or were homogenized with 1.0 ml. 60% ethanol (vide infra). The incubation medium was removed from the main compartment of the Warburg vessel with a Pasteur pipette and stored at -18°C until spotted on monodimensional chromatograms for measurement of the ^{14}C-lactate released during the incubation.

(xi) Measurement of Total Glycogen :

Method A was adapted from Walaas and Walaas [163]. The hemi-diaphragms in 0.4 ml. 30% KOH were digested at 95-100°C for 30 minutes with periodic mixing. (Evaporation of water during the digestion was controlled by the use of glass bubbles on each tube). The tubes were cooled and 0.1 ml. of 2% Na_2SO_4 was added,

followed by ethanol (2 ml.) to a final concentration of 80%. The contents of the tube were well mixed and left at 5°C overnight. The resulting precipitate was centrifuged using the highest speed of a bench centrifuge, the supernatant discarded and the precipitate resuspended in first 0.5 ml. 60% EtOH, then 0.5 ml. 70% EtOH, and finally 0.5 ml. 80% EtOH. The washed precipitate was finally digested in 0.4 ml. of 2N HCl for 3 hours at 95-100°C. The solution was put over dry KOH for 24 hours to remove the HCl; the precipitate, resuspended in 0.5 ml. water, was counted directly.

Method B This was a modification of the method of Wardlaw[114] and differed from method A in the addition of (a) 10 mg. oyster glycogen to facilitate the total recovery of the ^{14}C incorporated into glycogen; (b) 0.2 ml. 2% Na_2SO_4;

(c) centrifugation at 1500 R.P.M. for 30 minutes and (d) the inclusion of only one wash (with 1 ml. 80% EtOH) of the surface of the glycogen precipitate. The hydrolysate of the precipitate was neutralised with NaOH in the presence of 2 drops of BDH Universal Indicator before an aliquot was taken for counting.

(xii) Chromatography of Tissue Extracts and Measurement of Radioactive Metabolites including "Soluble" Glycogen and Oligosaccharides (Method C) :

The method of preparation of tissue extracts after incubation and the chromatography methods have been previously described[107].

The washed tissue was homogenised in 1.0 ml. 60% EtOH with a Vortex homogeniser, the blades of which were washed after each homogenisation with two aliquots of 0.5 ml. 60% EtOH and stored overnight at 5°C. The homogenate was centrifuged the next

day. The precipitate was resuspended in 1.0 ml. 60% EtOH, centrifuged and re-extracted twice with 1 ml. of water, the washings pooled, added to the supernatant and evaporated to dryness by vacuum suction at 40°C. The evaporated residue was made up to 0.5 ml. with water, any remaining insoluble material was centrifuged down and an aliquot (10µl of medium for monodimensional chromatograms, 50µl of tissue extract for bidimensional and 20µl for monodimensional chromatograms) of the supernatant taken for chromatography. This last water insoluble material was counted separately after its solubilisation by incubation with 0.5 ml. Hyamine at 40-50°C for 24-48 hours.

The total activity of the extract was determined by counting 20µl of the extract prior to "spotting" an aliquot. Between 4 and 6 "spots" were run on each sheet of Whatman No.1 filter paper for monodimensional chromatograms. The spots were applied with an Agla micrometer syringe very slowly in 2µl aliquots over a period of 1-2 hours; drying was assisted with portable fans and hair-driers placed under the spot of application.

(xiii) Chromatographic Methods :

Bidimensional chromatograms were run at room temperature using butanol:acetic:H_2O (v/v 40:11:25) for the first separation and tertiary-butanol:H_2O:picric acid (v/v 80:20:4g.) for the second separation[164]. The first separation generally ran for 16 hours and the second separation for 56-72 hours. A monodimensional and a bidimensional chromatogram were usually prepared of the same tissue extract. All results, with the exception of the incorporations into lactate, were scanned values

taken from the bidimensional chromatograms. Films of bidimensional chromatograms were made by leaving the chromatogram in contact with X-ray film (Kodirex 14" x 17") for 1-4 weeks.

(xiv) Automatic Scanning of the Radiochromatograms :

This was achieved by the use of automatic scanning equipment developed in the Biochemistry Department of Imperial College[165]. The counting efficiency of these machines is 11% and the reproducibility of the scanning results is ± 7%. The computer programme corrects for background emission, counting efficiency and variations between scanners, and the output expresses the radioactivity of separated spots on the radiochromatogram in μμc.

(xv) Recovery of Incorporated glucose-U-^{14}C :

About 75-100% of the glucose-^{14}C added to each vial could be wholly accounted for[107]. Analysis of the tissue extract by radiochromatography resulted in recovery of about 75% of the total radioactivity of the tissue extract by eluting the radioactivity from separated spots in monodimensional chromatograms and about 100-110% by automatic scanning of bidimensional radiochromatograms. The inefficiency in counting eluted radioactivity in the presence of paper shreds by a liquid scintillation method probably accounts for the error in the former method, while inaccuracies in the latter method are within the error involved in the transference of numerous small volumes of extract on to paper[107].

(xvi) Measurement of Incorporation of Medium glucose-U-^{14}C into (a) Soluble Glycogen and Oligosaccharides, and "Intracellular" Glucose :

The radioactivity in the chromatographically separated glucose and glycogen spots (including the oligosaccharide spots) was less when measured by shredding the traced spot on the chromatography paper with direct counting in a liquid scintillant after solubilisation with 1 ml. water compared with the radioactivity measured by the autoradiochromatographic scanner from bidimensional chromatograms. The glycogen and oligosaccharide spots were 84.6 ± 4.0% of the scanned values (for 39 extracts), while the glucose spot was 89.5 ± 2.6% (for 36 extracts). The reason for this was not clear; it is possible that there was some uncorrected inefficiency in the counting process owing to the presence of paper shreds or the tracing from film to paper may not have included a penumbra of radioactivity surrounding a labelled "spot" or the scanner may have overestimated counts from spots which were too close together and/which would not have been easily differentiated by the computer programme. Insulin increased the incorporation of glucose-U-^{14}C into glycogen, oligosaccharides and maltose[107]. In order to simplify the presentation of results showing the insulin effect on these substances, these values have been pooled. Soluble glycogen and oligosaccharides therefore refers to polysaccharide material extractable with aqueous ethanol, stable at 40°C, which when separated chromatographically, produces a pattern identical with the running of glycogen, maltotetraose, maltotriose and maltose (Fig.6). It does not include quantities of these substances found in the medium at the end of the incubation - (this was particularly true of liver slices).

(b) "Insoluble Glycogen" :

The residue insoluble in aqueous ethanol was largely glycogen as it could be extracted with hot alkali and hydrolysed to glucose by boiling in 2NHCl. The radioactivity not extracted by hot alkali was about 15% of the total radioactivity of the residue. The radioactivity extracted by hot alkali but not hydrolysed by 2NHCl was about 5% of the total radioactivity of the residue. These small percentages not extracted by hot alkali or boiling in 2NHCl were mainly protein. After these initial studies, the insoluble residue was routinely counted by solubilising it in 0.5 ml. Hyamine at 40-50°C for 24-48 hours and counting this in 10 ml. scintillant.

(c) Lactate :

Tissue and medium lactate values were pooled and referred to as total lactate. They were obtained from the mono-dimensional chromatograms. After the 16 hour separation, the paper was suspended in a tank containing 0.880 NH_3 in order to convert lactic acid to its less volatile ammonium salt.* The paper was then dried at room temperature and placed in close contact with X-ray film. After 24-48 hours the position of the lactate spot could be visualised, traced back on to the radio-active paper and the spot cut out, shredded and eluted in a counting vial with 1 ml. of water. 10 ml. of scintillant was added and the vial counted as described below. The medium lactate, obtained in the same way by separation from glucose-C^{14} present in the medium, was added to the tissue lactate to obtain a value for the incorporation into total lactate.

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* Using this technique quantitative recovery of ^{14}C-lactate has been achieved[166].

(d) Carbon dioxide : was collected during the incubation in 0.15 ml. Hyamine on a piece of fluted filter paper in the centre well of the Warburg flask and was transferred with methanol washings into 10 ml. of scintillation fluid.

(e) Medium glucose-^{14}C : an aliquot of which was shown by mono-dimensional chromatography to consist almost entirely of glucose-^{14}C, was counted directly in 10 ml. of scintillant.

(xvii) Use of Quarterdiaphragms instead of Hemidiaphragms :

It has been previously shown that the insulin effect on glucose utilisation or incorporation into glycogen was no different[114] or less[109] in quarterdiaphragms compared to hemidiaphragms. In the absence of insulin, these parameters were no different[114] or smaller[109] in hemidiaphragms compared to quarterdiaphragms.

It has been shown that the K^+ content of the medium slowly increases with time when quarterdiaphragms are used, but decreases with hemidiaphragms[167]. One would expect greater cellular damage (and therefore K^+ leakage out of the cell) with quarterdiaphragms than with hemidiaphragms, but the fact that the same authors found these quarterdiaphragms from fasted mice sensitive to insulin at 5×10^{-6} U/ml must indicate that the insulin response system was little affected, although the effects of changes of medium K^+ concentration on the sensitivity and response to insulin of quarterdiaphragms were not studied.

Quarterdiaphragms were preferred to hemidiaphragms in the insulin dose-response experiments as three insulin concentrations could be tested on each diaphragm. This economy was

particularly useful as obese mice on a restricted diet were not in abundant supply at the time.

Epididymal Fat Pads

(xviii). Removal of Epididymal Fat Pads :

After removal of the diaphragm (v.s.) the epididymal fat pads were removed from the animal's abdominal cavity with minimal trauma[105] by sectioning the pad, which was held up by its tip, at the point where the epididymal vessels join the internal spermatic artery and vein. This obviated any possibility of the inclusion of fragments of testis or epididymis with the adipose tissue. It is likely that the obese tissue received more handling than its lean counterpart. This was partly because each obese fat pad was divided into a large number of smaller pieces of approximately the same weight as an intact lean fat pad and partly because the oily leaking surfaces of these cut pieces added to their fragility. This was an important detail experimentally as it had been shown that excessive handling of epididymal fat pads in the rat markedly reduced the insulin effec which was sometimes barely significant[105]. The excised tissue was placed immediately in isotonic Krebs-Ringer bicarbonate buffer, pH 7.4, continually gassed with 95%O_2:5%CO_2 at room temperature - (chilling reduces the insulin effect[105]) - and blood and oil droplets were washed off the pad with gentle shaking.

After an interval of between 1-3 minutes, during which time diaphragm muscle was being washed or weighed, the fat pads were then blotted on filter paper and approximately 50-200 mg. samples were quickly weighed on a torsion balance and immediately placed in a vial (containing 2 ml. of gassed incubation medium) which was immediately stoppered. Paired pieces of epididymal fat

pad did not differ in weight by more than 50 mg. Ten pieces of adipose tissue (5 from each pad) were taken from each ob/ob mouse and in order to enable the weighing of these ten pieces to proceed as rapidly as possible two torsion balances with two operators were used concurrently. With this assistance the ten pieces could be weighed and distributed into vials in the space of 2 minutes which was about the time a single operator would have taken to weigh and distribute the 2 (sometimes 4) pieces of tissue normally obtained from a lean mouse. Because of the added time in adjusting the tare with pads from ob/ob-RD mice, there was some delay between the weighing of the first and tenth pieces of tissue from each pad of these mice. However, the pieces of tissue were always randomly distributed between control and insulin vials so that any effect that delay in weighing tissue might have had would not have affected the results on insulin sensitivity and response, even when expressed as paired increments of the values without insulin.

(xix) The Incubation Medium for Epididymal Fat Pads :

1.6 ml. of Krebs-Ringer bicarbonate buffer (125% hypertonic solution) with 5% dialysed crystalline bovine albumin. Sufficient glucose was added to achieve the desired final concentration of about 0.1%.

0.2 ml. 0.2% dialysed crystalline bovine albumin or insulin in 0.2% albumin.

0.2 ml. Glucose-1-^{14}C ($\simeq$0.5 - 1.0μc) in water

2.0 ml. Total volume of incubation medium.

(xx) Incubation Procedure :

One (or occasionally two) pieces from each fat pad of a lean mouse were incubated in the absence and presence of insulin. Of the ten pieces from each ob/ob or ob/ob-RD mouse, three were incubated in the absence of insulin and the rest with different concentrations of insulin. The concentrations of insulin studied are indicated in the results. The individually weighed pieces of adipose tissue were transferred to glass vials containing 2.0 ml. Krebs-Ringer bicarbonate medium[228], glucose and glucose-1-^{14}C, 4% dialysed albumin, with or without insulin. The bicarbonate buffer was gassed with 95%O_2:5%CO_2 for at least 10 minutes prior to receiving the adipose tissue, after which the vial was rapidly closed with a rubber Suba-Seal stopper with the attached glass cup for the collection of the $^{14}CO_2$ produced during the incubation, and incubated at 37°C for 2 hours in a metabolic shaker at 78 C.P.M.: (the rate of glucose oxidation is not linear after three hours[105]).

(xxi) Extraction of Lipid :

After incubation, the omental fat tissue was washed and drained on filter paper three times in saline before being homogenised in 5 ml. $CHCl_3$:CH_3OH(v/v2:1) in an ice-bath. The homogenate was centrifuged and 4 ml. of the supernatant was pipetted out and shaken with 1 ml. of 0.73% $MgCl_2$ solution. 2 ml. of the chloroform layer was taken for counting.

Isolated Adipocytes from Epididymal Fat Pads

(xxii) Siliconisation of Glass Vessels :

Silicone liquid was poured into or through any glass flasks, funnels or microscope slides used in isolated adipocyte

experiments; these were then baked at 200°C for at least 3 hours or at 110°C for at least 3 days.

(xxiii)Collagenase :

The collagenase present in filtrates of Cl. histolyticum is specific for collagen and acts at physiological pH. It was first used in 1964 in an unpurified form[136] for the preparation of rat adipocytes. Chromatographically pure collagenase (usually the type b enzyme) is now commercially available and has been shown to contain little or no peptidase activity. Further, it does not act appreciably upon elastin, fibrin, keratin, casein, haemoglobin or albumin[168]. However, the more widely used unpurified enzyme is known to contain a peptidase and a trypsin-like proteinase. In view of the ability of trypsin to destroy the responsiveness to insulin of isolated fat cells of the rat[169], it is surprising that such consistent results using impure collagenase and isolated rat adipocytes have been widely obtained. It had been reported that fat cells prepared according to Rodbell[136] and using crude collagenase were completely permeable to glucose[170]. However, Lech et al.[171], in one of the very few investigations citing the use of chromatographically pure collagenase, found that rat adipocytes prepared with the pure enzyme showed normal osmotic behaviour when placed in mediums of varying tonicity. Further, they recorded no adverse effect in the stimulation of lipogenesis from glucose-U-^{14}C in these cells by insulin. Unpurified collagenase is also known to destroy the basement membrane of fat cells[172] and of the sarcolemma of muscle[173]. Although it is not known whether chromatographically pure collagenase also destroys the basement membrane, and because comparative studies using the pure and impure enzyme were not attempted, the chromatographically pure enzyme was chosen for the work reported

here in an attempt to preserve as much as possible of the native composition of the responding adipocyte plasma membrane.

(xxiv) Preparation of Isolated Mouse Adipocytes :

The method has been adapted from that described by Rodbell.[136] For each adipocyte suspension, between ten and twenty lean mice or 1-3 ob/ob or ob/ob-RD mice were decapitated and their epididymal fat pads rapidly and carefully removed and placed in a Petri dish containing an isotonic solution of bicarbonate buffer, with 0.005% glucose and no albumin, under a stream of 95%O_2:5%CO_2 gas at room temperature. The tissue was divided into fragments of about 50 mg. wet weight and transferred, without weighing or blotting to a siliconised 25 ml. Quickfit Erlenmeyer flask containing 2-3 mg. chromatographically pure collagenase in 3-5 ml. of the same bicarbonate buffer without albumin. The flask was then placed in a water bath at 37°C and vigorously shaken (120-160 C.P.M.) for 30-45 minutes by which time visible disintegration of the fragments was evident. The flask was removed from the water bath and its contents filtered through a nylon mesh[174], (pore size 250μ, "Nybolt", from H. Simon Ltd., Stockport, Cheshire), into 2-4 cellulose nitrate centrifuge tubes ($\frac{5}{8}$" x 3" each) (Beckman Inc., Palo Alto, Calif., U.S.A.). Gentle stirring with polyethylene tubing or a stainless-steel spatula was often needed to break up fragments that had still retained some tissue structure. The resulting creamy suspension was centrifuged for about a minute in a bench centrifuge at a very low g (the centrifuge was turned on and off at its lowest setting so as to prevent the centrifuge achieving the maximum speed of the lowest setting). The bottom of the cellulose nitrate centrifuge tube was then pierced

to let out the clear infranatant and the layer of adipocytes was prevented from passing out by pushing another intact cellulose nitrate tube over the bottom of the punctured tube. 10-12 ml. of fully gassed bicarbonate buffer without albumin was poured over the cells, and the suspension mixed by pouring into another intact tube and the resuspended cells centrifuged again. This washing procedure was carried out three or four times. It is an important step in the preparation of mouse and rat [93] adipocyte suspensions: insufficient washing of mouse adipocytes was on some occasions suspected as being responsible for suspensions that failed to respond to insulin. This might have been caused by endogenous insulin or insulin-inhibitory factors bound to the adipocytes which would have been removed by further washing, or more probably destroyed more effectively by fat cells without a basement membrane[104]. It was also possible that increased washing in an albumin-free buffer reduced the number of intact cells so that an optimum cell:insulin ratio was established. A high concentration of fat cells (300 mg. of fat cells/ml. medium) is known to inactivate insulin sufficiently to affect its bioassay by isolated adipocytes[104]. The colour of the suspension usually changed from the yellow due to contaminating red blood cells to a clean white by the end of the third wash. The total time taken to prepare and wash the cells seldom exceeded 25 minutes. This washed suspension was then transferred to a plastic beaker and made up to the volume desired with gassed isotonic bicarbonate buffer. A photograph of such suspensions prepared from lean mice & fixed with glutaraldehyde:trichloroacetic acid:water (v/v 25:1:400) after filtering through a 30μ nylon mesh is shown in Plate 2. Cell Lysis: When required, cell suspensions were lysed using a M.S.E. ultrasonicator[175].

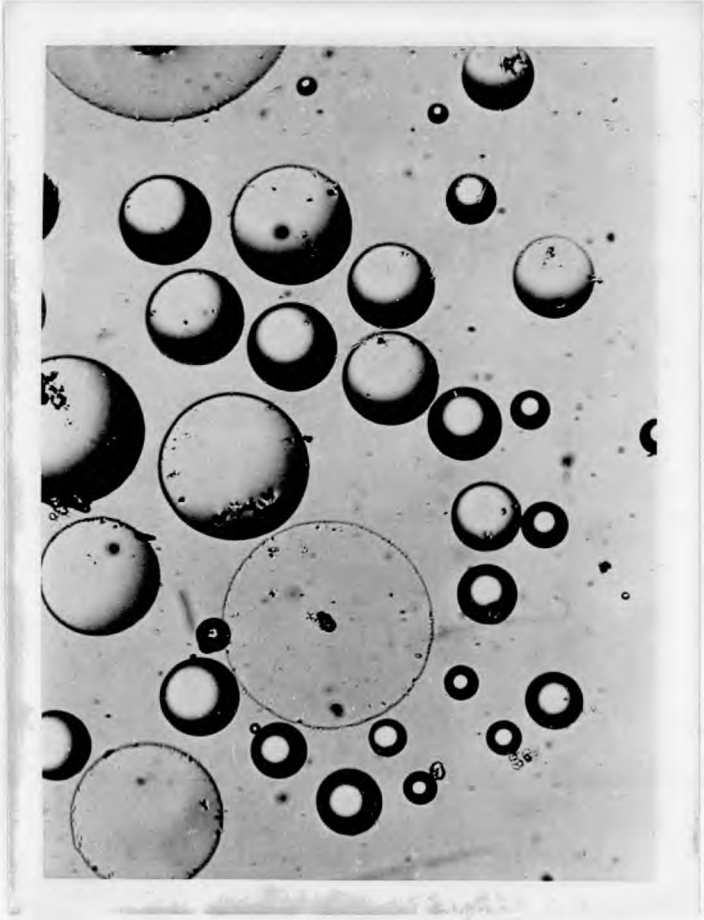


Plate 2: Trichloroacetic acid-glutaraldehyde fixed cells. Adipocytes from the epididymal fat pads of 6-8 month old lean mice after filtering with a silver mesh (pore size 30μ) and fixation with trichloroacetic acid-glutaraldehyde. It can be seen that oil droplets do not possess the high refractive index of the intact adipocytes. The preparation after filtering was relatively clean of broken membrane fragments.
Magnification x 210.

(xxv) Incubation Procedure :

The cell suspension was continually gassed at room temperature while being pipetted out into vials. It has been shown that temperature reduction during the preparation of adipocytes has little effect on their subsequent behaviour at 37°C [93]. After firm shaking of the suspension to homogenise the distribution of the cells, 0.8 ml. was drawn up into a 1 ml. plastic disposable syringe fitted with an appropriate size of polythene tubing ½cm. in diameter and about 10-15 cm. long. This permitted the rapid dispensing of 0.8 ml. aliquots of the suspension into the plastic vials used for all incubations of adipocytes. The adipocyte suspension had to be pipetted out as rapidly as possible as mouse adipocytes had a tendency to stick to the sides and bottom of the plastic beaker; this tendency was not noted with suspensions of rat adipocytes and as it limited the distribution time of the suspension, the number of vials studied per adipocyte suspension never exceeded 50. Changing the concentrations of Ca^{++} and Mg^{++} or using Teflon-coated plastic beakers did not significantly help to solve this problem. Cell breakage also tended to occur at this stage and it is probable that the presence of albumin in the medium as fatty acid acceptor stabilised the preparation as it was shown that once in the albumin-containing incubation medium, rates of oxidation of glucose-1-^{14}C were linear (see Fig. 13). The plastic vials, (obtained from the Packard Instrument Co.), contained the following prior to the reception of the fat cells:

0.8 ml. 10% dialysed, crystalline albumin in 150% hypertonic bicarbonate buffer containing sufficient glucose to achieve the final concentration required.

0.2 ml. 0.2% dialysed crystalline albumin solution or insulin at different concentrations in 0.2% dialysed crystalline albumin solution.

0.2 ml. glucose-1-^{14}C of low specific activity 0.5 - 1.5μc in water.

The vials were arranged under a gas manifold so that the incubation medium was gassed with 95%O_2;5%CO_2 for at least 10 minutes prior to the addition of the 0.8 ml. aliquots of cell suspension (in isotonic bicarbonate buffer without albumin but containing 0.005% glucose). The final pH after the addition of the cells was 7.4. The vials were immediately stoppered with rubber Suba Seals fitted with hanging glass wells for CO_2 collection (v.i.) and placed in a water bath at 37°C. Incubations were carried out for 60 minutes (although results from a few incubations of 120 minutes have also been included) with gentle shaking (78 C.P.M.), (although similar rates of oxidation of glucose-1-^{14}C were obtained with no shaking), and the $^{14}CO_2$ collected at the end of the incubation was treated as described below.

(xxvi) Collection of CO_2 in Adipose Tissue Experiments :

For the collection of CO_2 produced during the incubation period a small glass cup containing a piece of fluted filter paper (1 x 2 cm. Whatman No.1) was suspended in the vial by means of a spiral wire which was held in the Suba-Seal rubber stopper[176]. At the end of the incubation period, the vials were placed in an ice bath, and 0.2 ml. of ethanolamine: methanol (1:1 v/v) was injected into the glass cup to absorb the CO_2

released by the 1 ml. of 2N HCl injected into the incubation medium; both solutions were carefully introduced into the correct compartments, without opening the vials, by means of syringe needles inserted through the rubber stopper. The vials were then shaken again at 37°C for 30 minutes or left overnight at room temperature. Neither procedure affected the recovery of $^{14}CO_2$ from $Na_2{}^{14}CO_3$ which, as can be seen in Table 4, was about 94%.

The $^{14}CO_2$ collected after the incubation of fat pads or adipocytes was counted by gently detaching the glass cups from the wire holders and tipping them, with the fluted filter paper and ethanolamine:methanol contents, into glass counting vials (Packard Co. Ltd.) and adding 20 ml. of scintillation fluid, (see Radioactive Measurements). The vials were then counted in the liquid scintillation spectrophotometer.

TABLE 4 : Recovery of $^{14}CO_2$ from $Na_2{}^{14}CO_2$

0.2 ml. ethanolamine:methanol (1:1) on fluted filter paper in glass cup

1.0 ml. 2N HCl added to incubation medium.

$Na_2{}^{14}CO_3$ added $\mu\mu c$	$^{14}CO_2$ recovered $\mu\mu c$
376702	352690
389660	371482
403683	369949
Mean 390015±7791 (3)	364707±6025 (3)

Recovery = 93.5%

(xxvii) Radioactive Measurements :

^{14}C Counting Methods

Liquid scintillation methods were used to count ^{14}C. Most of the work was done using a modified PBD scintillant[177] but the earlier work was counted using Bray's scintillant[178]. 10 ml. of Bray's or 20 ml. of a 2:1 mixture of PBD:methyl cellosolve were added to each glass counting vial (Packard) containing either ^{14}C glucose or ^{14}C lactate in water or the glass cup and fluted paper holding the ethanolamine: methanol in which $^{14}CO2$ had been collected during the incubation. The vials containing the glass cups etc. were left at room temperature for 24 hours and then placed in a Beckman or Packard Liquid Scintillation Spectrophotometer and counted for 10 or 20 minutes. The accuracy of the counting was generally between 2-5%. The efficiency of counting was determined by the channels-ratio method[179] using internal standards, and the results calculated in $\mu\mu c$. Quenching was corrected for by the addition of internal standards.

^{125}I Counting

^{125}I was counted directly in a gamma-counter, (Tracerlab Instruments Ltd.) Background emissions were subtracted from all readings.

(xxviii) Method of Calculating Results :

All results were expressed as $m\mu M$ glucose incorporated into glycogen or oxidised to $^{14}CO_2$ by dividing the $\mu\mu c$ of glycogen (as glucose) or $^{14}CO_2$ obtained by the specific activity of the medium of that experiment, viz. the radioactivity present in each vial in $\mu\mu c$ per μM glucose present in the tracer and unlabelled glucose added to achieve a required final

concentration. This method of expressing the results assumes that the specific activity of the metabolic product measured with and without insulin is the same as that of the glucose substrate, or differs equally in lean and ob/ob mice. The incorporations and oxidations were then further divided by the wet weight of the tissue and expressed finally as mμM glucose/60 min. or 90 min./mg. wet weight tissue.

The insulin effect was measured either as the difference between the value of the insulin-exposed pad and the paired control pad[105], viz. the increment in incorporation, or oxidation, or as this increment expressed as a percentage of the value without insulin. This latter form of expression was used for all experiments with adipocyte suspensions. Each dose-response curve shown in Figs.14(a)(b) and 15(a) (b) was derived from one cell suspension. The response at each insulin concentration was the mean of 3-4 determinations and had a mean s.e.d.m. of ± 12%.

ADIPOSE TISSUE AND CARCASS MEASUREMENTS

(xxix)Total Nitrogen Estimation :

Complete digestion of the adipose tissue was effected by refluxing the pad with a sulphuric acid-perchloric acid mixture (v/v 45:1) containing 0.3% SiO_2. The NH_3 produced was measured in an auto-analyser using a colorimetric method by which the NH_3 was converted into a blue compound of the indophenol type by an alkaline phenol/hypochlorite system and was read at 630 mμ wavelength. Ammonium sulphate solution was used as the standard.

Pieces of tissue smaller than 300 mg. were found to give significantly higher values of nitrogen per unit wet weight of tissue (Table 20) but consistent values were obtained for tissue samples heavier than this. It is suspected that the blotting procedure was more efficient for smaller pieces of tissue, thus reducing proportionally the dry/wet weight ratio. It is relevant to recall here that glucose metabolism (per unit wet weight) of epididymal fat pad shows an inverse relation to the wet weight of tissue used, particularly with pieces of tissue weighing less than 70mg/ [122,126] For this reason, the nitrogen values have been separated into groups from pieces less than, and more than, 300 mg.in wet weight.

(xxx) Total Water Content :

The fat pads were placed in tared vessels, weighed and then heated in an oven at 35-40°C until no further reduction in weight occurred.

(xxxi) ^{3}H-Sorbitol Space :

Each fat pad was incubated for 60 minutes, as described previously, with 0.5 μc ^{3}H-Sorbitol and 1 mg. Sorbitol, but no ^{14}C glucose. At the end of the incubation, the pad was washed and blotted on filter paper three times before being homogenised in 1 ml. 6% perchloric acid. The precipitate was spun down at 2000 R.P.M. for 20 minutes and the supernatant, after neutralisation with 30% KOH in the presence of BDH Universal indicator, was made up to 5 ml. in a volumetric flask and 1 ml. aliquots were taken for counting. The ^{3}H-Sorbitol space in μl/mg. wet weight of pad was calculated from the following formula:

$$^{3}\text{H-Sorbitol space } \mu\text{l/mg.} = \frac{\text{Tissue } \mu\mu\text{c } ^{3}\text{H-Sorbitol/mg.}}{\text{Medium } \mu\mu\text{c } ^{3}\text{H-Sorbitol/}\mu\text{l.}}$$

(xxxii) Coulter Counting and Sizing of OsO_4 Fixed Fat Cells From Suspensions and in Intact Tissue:

The number of adipocytes per mg.wet weight of epididymal fat pad was estimated by fixing adipocytes in OsO_4 and counting them, using method III as described by Hirsch and Gallian[180a]. 20-50 mg. pieces of fat pad were excised, weighed and placed directly in 2%OsO_4 in 0.05M collidine:0.15M NaCl buffer pH7.4. Fixation at 37°C for 24 hrs. and at room temperature for 1-2 weeks in a fume cupboard resulted in the complete fixation of the tissue. The fixed cells were filtered through a nylon screen of 250μ pore size before being trapped by a nylon mesh of 20μ pore size, (H.Simon Ltd., Stockport, Cheshire). The washed, fixed cells shown in Plates 3(a)(b) were then suspended in 50-80 ml. of clear glycerol:0.9% NaCl(v/v 1:1)[180b], so as to increase the time during which heavier fixed cells remained homogeneously suspended while being counted. 0.5ml. aliquots of the fixed cell suspension, containing between 100-400 cells each,were counted in a Coulter Counter Model A(by courtesy of the Pathology Dept., Hospital for Sick Children, Great Ormond St., London, W.1. Coincidence errors at this dilution using a 400μ aperture were allowed for. The average of 6-8 readings was taken, multiplied by the dilution used and expressed per mg.wet weight of tissue. The average S.E.M. of 6-8 determinations of the counts in aliquot from each suspension was ± 2.4% of the number of cells per mg.wet weight of epididymal fat pad for 20 fixed suspensions. Adipocyte suspensions prepared by digestion with pure collagenase were also fixed with 2%OsO_4 for 3-4 hrs. By careful dispersal of the washed, fixed adipocytes on a siliconised glass slide,

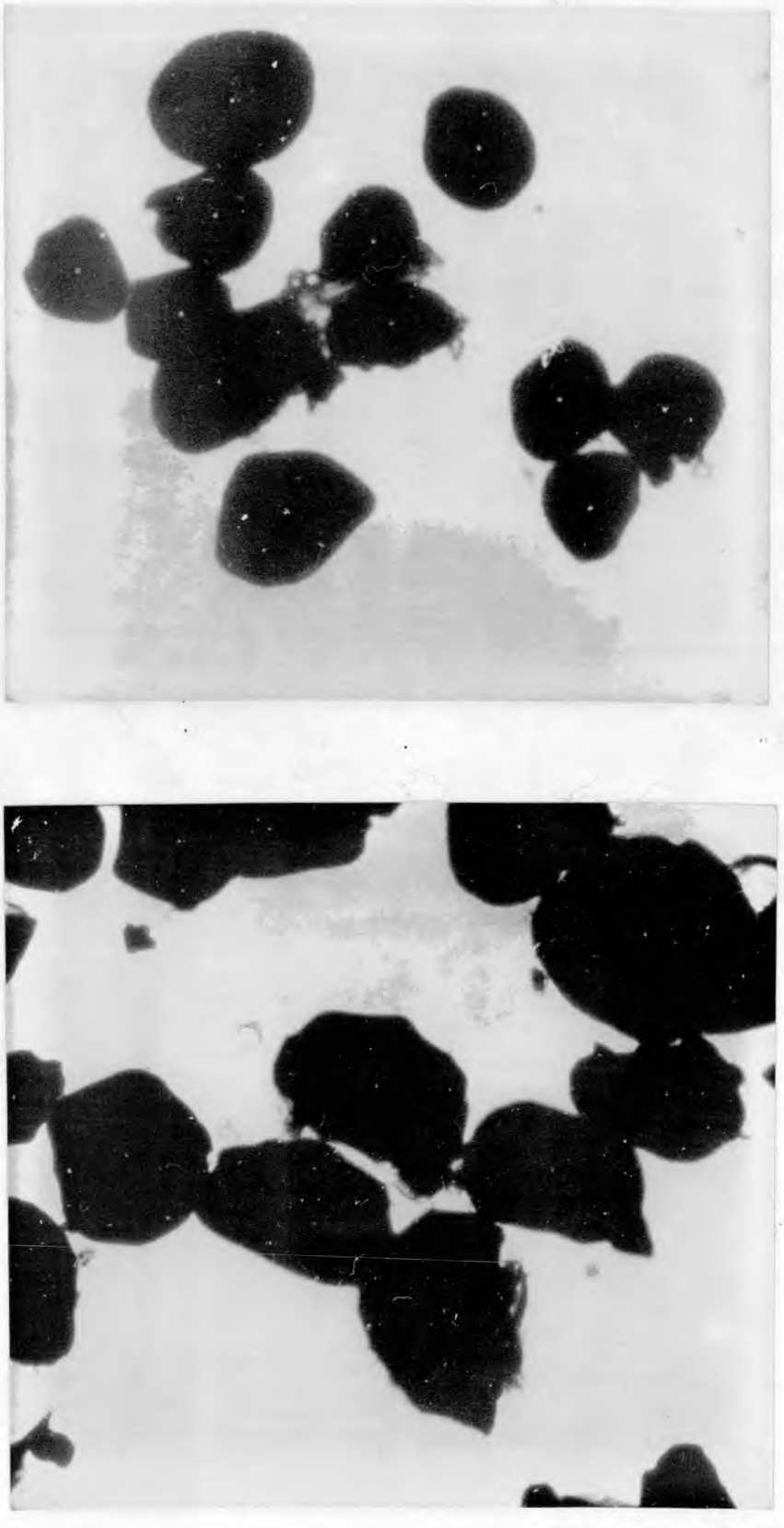


Plate 3(a)(b): Adipocytes from 2-4 month old lean (upper) and ob/ob mice (lower) fixed with 2%OsO_4 by direct tissue fixation (Method III of Hirsch and Gallian 1968)[180a]. Magnification x 105.

clumping could be avoided and microphotographs taken for cell sizing. Photographs of adipocyte suspensions from lean and ob/ob mice, fixed just prior to incubation are shown in Plates 4(a)(b).

The sizing of the cells was done with a Zeiss Particle Size Analyser TGZ3, (by courtesy of The Central Polytechnic, Regent Street, London, W.1), from magnified photomicrographs of the cell suspensions. Cells were sized by ascertaining the magnification of the optical system used (objective x2.5, tube factor x1.25, camera factor $\frac{10}{3.2}$ and negative enlargement x10.7 = total magnification, x105) and checking this by photographing a micrometer slide under identical conditions. Sizing of the cell diameter of the deformed adipocytes prepared by complete OsO_4 digestion was estimated by aligning the light disc of the particle size analyser to the "average" diameter judged subjectively by eye. The sizing of both the deformed adipocytes and the spherical fixed adipocytes after collagenase digestion were therefore both visual estimates, but because of the circular outline of the light source used for the sizing, the estimations of the spherical particles were likely to be more accurate.

(xxxiii) Measurement of Plasma Volume and the In Vivo ^{125}I-Albumin Space:

The volume of distribution of ^{125}I-Albumin was used as an index of plasma volume[181]. It is known that albumin distributes itself in part of the lymphatic system as well as occupying the plasma vascular compartment, but assuming no great difference between lean and obese mice in this respect, the use of this tracer is quite suitable. 20µl of ^{125}I-human serum Albumin in 0.75% NaCl and 0.9% benzyl alcohol (1µc of a 2% solution) was carefully injected with a Hamilton microlitre syringe into the femoral or portal vein or the inferior vena cava of a lean or obese mouse previously anaesthetized with 0.05 - 0.10 ml. Nembutal. Very fine needles (30G x ½") were used for this injection so as to minimise leakage from the site of injection. A small chip of tissue paper was used to trap any extravascular radioactivity and this was subtracted from the known counts expelled by this syringe, set at 20µl., to give the actual counts of ^{125}I-Albumin injected intravascularly. Early experiments showed that equilibration of the tracer was complete between 15-25 minutes, so sampling of the diluted tracer was started 20 minutes after the initial injection by aspirating

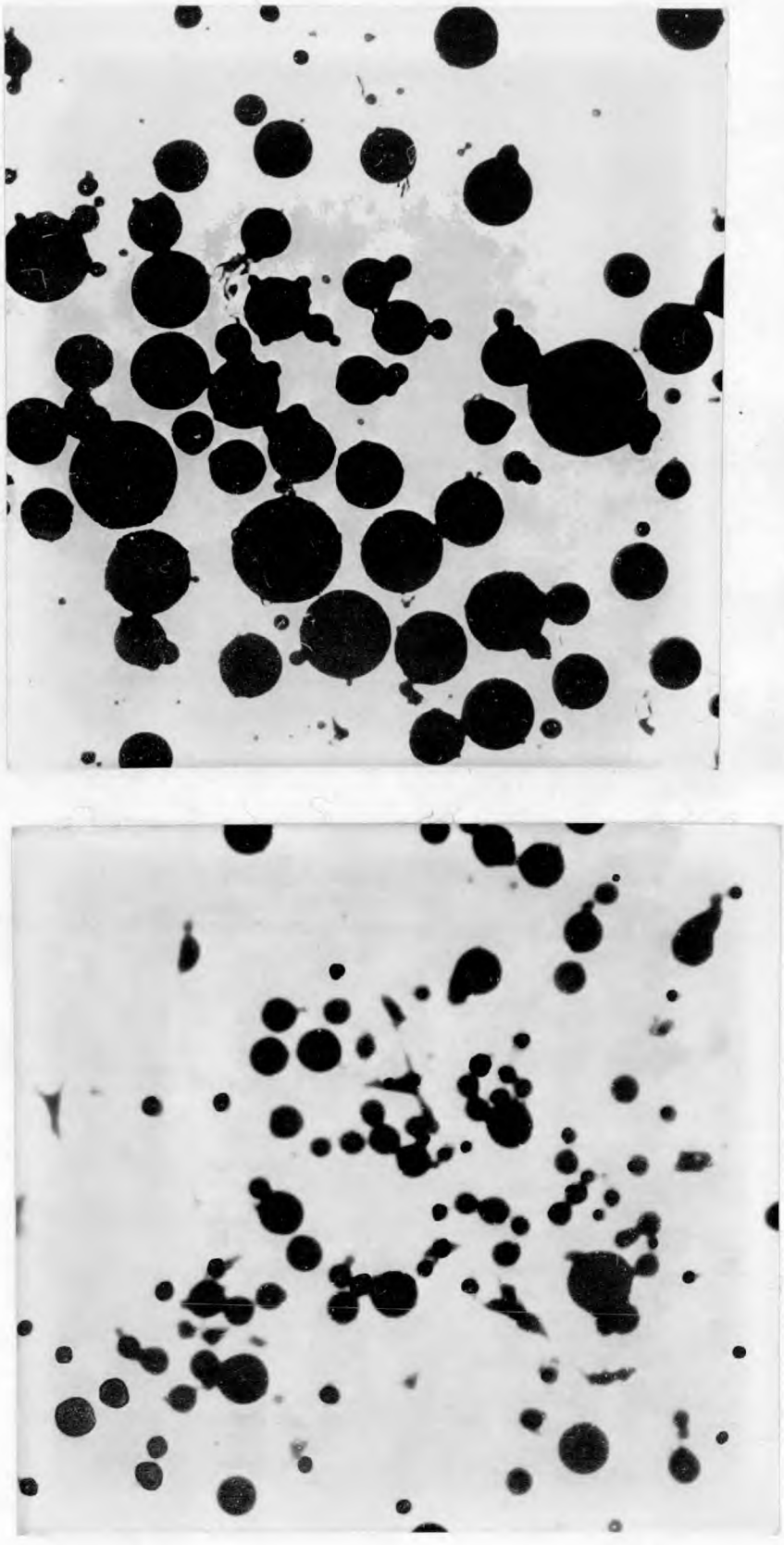


Plate 4(a)(b): Adipocytes of 2-4 month old lean (upper) and ob/ob mice (lower) partially fixed with 2%OsO_4 after preparation of isolated adipocytes by collagenase digestion (method of Rodbell[136]). Magnification x 105.

blood from the heart. During the 20 minute period of mixing, the abdomen was closed with a clip and the animal placed on a tray under a strong light, suitably adjusted to keep the animals warm. The animals did not regain consciousness at any time during the experiment. After collection of the blood the animals were killed by cervical dislocation and the two fat pads were rapidly excised and chilled with clamps frozen in liquid nitrogen, before being weighed. It was not found necessary to devise a more sophisticated method for the separation of the fat pad vascular compartment from its origin from the internal spermatic artery. The tissue consistently appeared bloodless and there was never any loss of blood from the cut end. Each weighed pad was homogenized in concentrated Teepol in the presence of a silicone antifoam agent. After making up to volume in a 20 ml. volumetric flask, a 5 ml. aliquot was taken for counting in a Tracerlab gamma-counter. The efficiency of counting ^{125}I-Albumin in 5 ml. of Teepol was found to be 68% of the counts without Teepol and the counts of ^{125}I-Albumin in the Teepol homogenate were corrected for this error.
The plasma volume was calculated from the following relation:

$$\text{Plasma volume (ml.)} = \frac{\text{CPM injected}}{\text{CPM/ml. plasma after 20 mins.}}$$

where the CPM injected = the average of six aliquots expelled by the Hamilton syringe minus the CPM on tissue paper outside the injection site.

and the Fat Pad ^{125}I-Albumin space (μl/mg.) = $\frac{\text{CPM / pad} \times 10^3}{\text{CPM / ml. plasma from the same mouse} \times \text{wet weight in mg. of pad}}$

There was no significant difference in the plasma volume of lean mice when injected by any of the 3 routes mentioned, and no large statistical difference between fed and fasted obese mice. Accordingly, all values have been pooled.

(xxxiv)Method for Fat Extraction and Water Content Determination of Carcasses in lean, ob/ob and Goldthioglucose obese mice:

The fresh carcass was first weighed in a tared vessel, fragmented, and then homogenised thoroughly in water for about 5 minutes at room temperature so as to completely pulverise all tissues. The homogenate was next freeze-dried until no further change in weight occurred, the dried powder being weighed, and this weight, subtracted from the body weight enabled one to obtain the water content of the carcass.

The freeze-dried carcass powder was then extracted with about 200 ml. $CHCl_3$:CH_3OH(2:1), and well shaken with 40 ml. 0.73% $MgCl_2$; after filtering the mixture, the supernatant was removed when the phases had settled. The chloroform extract was dried down under reduced pressure at 35-40°C over a period of days until no further change in weight of its tared container occurred. The oily fat left behind was then weighed to give the fat content of the carcass.

The nitrogen content of the dried, fat-free residue was determined on an aliquot of the residue by the method described below.

IN VIVO EXPERIMENTS

(xxxv)Assay of Immunoreactive Insulin :

Insulin was assayed, with minor modifications, by the double antibody method of radioimmunoassay[182], using the kit supplied by the Radiochemical Centre, Amersham, England[183]. In this assay, ^{125}I-ox insulin competes with immunoreactive material in the sample for the insulin binding reagent, which contains guinea-pig antibodies to ox insulin and rabbit antibodies to guinea-pig serum proteins (the latter to precipitate the insulin-antibody complex). In the present method, the maximum counts bound (in the absence of unlabelled insulin immunoreactive material) amounted to about 30% of the total ^{125}I-ox insulin present in the mixture. The sample size was always 0.1 ml. and duplicate or triplicate determinations were carried out. ^{125}I was counted in a gamma-counter (Tracerlab Instruments Ltd.).

Most plasma samples from fed ob/ob mice required dilution (up to ten-fold) before assay in order to increase the counts/minute of ^{125}I-insulin bound to a satisfactory percentage (i.e to enter the middle range of the assay). In these cases the counts/minute bound were either corrected for dilution by use of the ob/ob plasma dilution curve (Fig.4) or, if dilution was threefold or less, and if percentage counts/minute bound was 20-35%, by reading directly from the "Standard" pancreatic extract curve.

Fig.4 shows the assay curve obtained with crystalline ox insulin, ob/ob mouse plasma and lean and ob/ob pancreatic extracts: (it should be noted that the curve for plasma has been arbitrarily superimposed on that from extracts

of pancreas by assuming that immunoreactive material from each tissue behaved similarly if plasma was undiluted). It was evident that the mouse pancreatic and plasma insulins behaved very differently in their binding affinities for the ox insulin antibodies than did ox insulin. Consequently the assay curve for ox insulin could not be used for assay of mouse plasma samples.

As standard mouse insulin or antibodies to mouse insulin were not commercially available (at the time this work started), and as biological assay of pancreatic extracts was expensive and time-consuming, ox insulin standard curves were obtained after each assay to test the reproducibility of the assay system which was extremely constant with any one batch of ^{125}I-ox insulin and insulin binding reagent.

Values for the immunoreactive insulin present in mouse plasma were obtained from the percentage of maximum counts minute of ^{125}I-ox insulin bound to insulin binding reagent by us of an assay curve of "standard" lean mouse pancreatic extract, (which extract had been bioassayed by the mouse convulsion assay v.i. Fig.4) and expressed in terms of this extract as μU/ml of "pancreatic" mouse insulin.

(xxxvi) Acid-Alcohol Extraction and Partial Purification of Mouse Insulin :

This method had been taken direct from Kenny [184] and was in most respects similar to other published procedures for extraction of insulin from pancreas.

Approximately 2 g. of (lean or ob/ob) mouse pancreas were homogenized with 6.0 ml. of ethanol:H_2O:HCl (v/v) (51:17:1) over ice. The homogenate, after standing for 2 hours

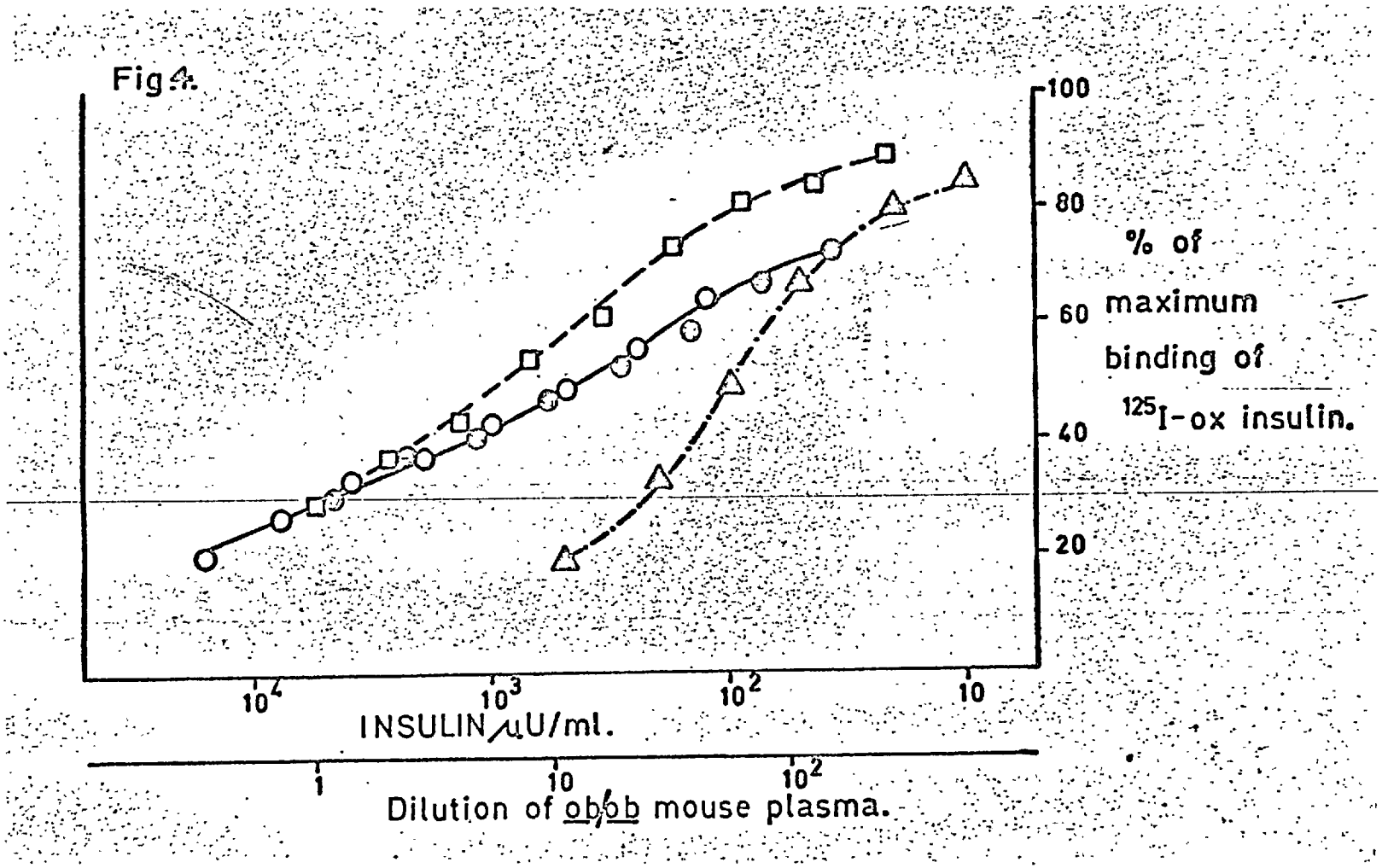


Fig. 4 : Binding of ^{125}I-ox insulin to antibody in the presence of crystalline ox insulin (△), different dilutions of mouse plasma (□ ; at 1 on the abscissa, pooled ob/ob plasma was undiluted) or pancreas extracts (o ,ob/ob; o,lean). Results are the average of two assays with different batches of ^{125}I-ox insulin. Further details are in the text.

at 5°C was centrifuged for 20 minutes at 2000 R.P.M. at 0°C. The resultant pellet was resuspended in 6.0 ml. acid alcohol, centrifuged again and the two supernatant extracts pooled, neutralised with concentrated NH_4OH and filtered into 50 ml. plastic centrifuge tubes.

To 9 ml. of the extract, 15 ml. EtOH and 25 ml. diethyl ether were added. After shaking this mixture, it was allowed to stand overnight at 5°C until the next morning when, after centrifugation at 2000 R.P.M. for 20 minutes at 0°C, the alcohol/ether supernatant was discarded. The insulin precipitate was partially dried in a stream of N_2 without heating but was stored at -18°C slightly damp so that the precipitate before use was gummy rather than a dried powder.

(xxxvii) Standard Solution of Mouse Insulin :

Pooled pancreas from fifteen 10 month old female lean mice (approximately 2 g. in all) was extracted with acid ethanol and a partially purified extract of insulin was obtained according to Kenny[184] (v.s.). The solid extract, which had been stored at -18°C, was taken up in 5 ml. 0.05NHCl and a bioassay for insulin-like activity by the mouse convulsion method was kindly performed, (against the 4th International insulin standard), at Burroughs-Wellcome Ltd., Dartford, Kent.. This "standard" solution was found to contain 6.2 U/ml. of insulin (fiducial limits $\pm$ 1U; p = 0.95). Samples of this solution and of other extracts of pancreas (one each from lean and ob/ob mice) were immunoassayed at various dilutions with two different batches of ^{125}I-ox insulin and the resulting assay curves plotted against the biological activity in IU

obtained by bioassay of the previous pancreatic extract of lean mice. The assay curves for lean and ob/ob mouse pancreatic extracts, shown in Fig. 4, could now be plotted on the same scale as the assay curve for ox insulin, as both insulins were assayed by the same mouse convulsion method at the same institution. Exact quantitative alignment, although suggested by both curves being on the same graph, is not possible as the ox insulin used (available as a crystalline, commercially available powder) was not bioassayed at the same time as the mouse pancreatic extracts. Further, the bioassay has been presumed to vary with concentration in a linear fashion, whereas the immunoassay of lean mouse pancreatic extract clearly does not (Fig. 4). Also it is clear (Fig. 4) that the difference between the ox insulin and mouse pancreatic extract curves was pronounced especially at high insulin concentrations. This has also been shown to occur with rat plasma; suboptimal precipitation caused by cross reaction between rat plasma gammaglobulins and rabbit anti-guinea-pig serum proteins has been suggested as a possible explanation[185]. Pancreatic extracts from lean and ob/ob mice, however, gave identical dilution curves, in agreement with the findings of others[46,48]. Dilution assay curves of ob/ob mouse plasma were possible because undiluted plasma from fed older ob/ob mice consistently gave ^{125}I-ox insulin binding percentages of below 30%. The specimen curve of ob/ob mouse plasma shown in Fig.4 has been arbitrarily superimposed on the plot for extracts of pancreas from lean and ob/ob mice at the point where the plasma was assayed undiluted, (as bioassay using the mouse convulsion method was not possible with the small quantities of insulin in lean or even ob/ob mouse plasma). It was assumed that immunoreactive material from

the pancreas and from undiluted plasma behaved similarly in the immunoassay, and that the biological activity of the IRI assayed for plasma was the same as that from pancreatic extract[48]. No comparable immunoassay curve for plasma from lean or ob/ob-RD mice was possible since not enough total immunoreactive material could be collected. Pancreatic extracts from lean and ob/ob mice were also assayed in this Department[186] by a single antibody method using ethanol to precipitate the insulin antibody complex; the dilution assay curves for both these extracts were identical by this method of assay as well.

Ob/ob plasma can be seen in Fig. 4 to have a slightly higher binding affinity for ox insulin antibodies than does ob/ob mouse pancreatic extract, (assuming its arbitrary position on the scale is correct). There are many possible reasons for this phenomenon, amongst which the most obvious, stated broadly, is the presence of a substance(s) in plasma which changes the energy of reaction of the binding of mouse insulin with ox insulin antibodies or of the double antibody complex formation, (or alternatively, the absence of such a substance in plasma but its presence in pancreatic extract). The most important variable to have caused this would be the presence of the normal constituents of plasma. The withdrawal of blood from the animal or its haemolysis may also have introduced interfering substances, or alternatively, trace substances introduced in the preparation of the pancreatic extract may have been involved. Similarly the lower binding affinity of pancreatic extracts might also be indicative of the presence of precursors of insulin (e.g. proinsulin) which have a greatly reduced binding affinity for insulin antibodies. As it was difficult to establish the

significance of this deviation, which was not big, and especially as it was not known if such a difference existed wit tissues from lean mice, it was felt that it was more reliable to use the curve obtained with lean and ob/ob mouse pancreatic extracts, (the curve was drawn through both lean and ob/ob mouse pancreatic extract curves), as the "standard" curve from which all values would be read.

(xxxviii) Sampling of Blood and Liver : Experiments were begun at 10.00 hours, unless indicated in the Tables.

The mice were anaesthetised with ether, the abdomen opened and the heart exposed after removal of the diaphragm. About 1.0 - 1.5 ml. blood was withdrawn into a syringe through a needle containing a negligible volume of heparin solution (1:25000). Heparinized blood was centrifuged without delay. Plasma was removed by suction and kept at 5°C. Determinations, particularly of glucose, were usually carried out immediately. Plasma was frozen if kept for more than a few hours.

(xxxix) Gastric Intubation of Mice :

The mice were lightly anaesthetised with ether; the stomach was intubated with firm, polythene tubing of about 1 mm. external bore. 0.5 - 1.0 ml. of either 0.9% NaCl or glucose of between 1-2M was injected intragastrically as indicated in the results. The mice were fully conscious within 2 minutes. In the event of subsequent intubations 90 minutes later, the mice were replaced in cages devoid of bedding.

(xl) Glucose Assay :

Glucose was determined on an Auto-Analyser by a glucose oxidase method[187] after blood had been deproteinised with equimolar amounts of barium chloride and zinc sulphate[188].

(xli) Statistical Analyses :

In keeping with previous recommendations on hormone assay[189,190], when more than one concentration of hormone was being tested, all observations including negative values, were considered. This was especially necessary in order to get a quantitative evaluation when there was no response to insulin. Preliminary calculations on the results of the incorporation of glucose-U-^{14}C into glycogen, the oxidation of glucose-1-^{14}C or the insulin effect on these parameters, suggested symmetrical distributions that were nearly always leptokurtic. It was therefore reasonable to present the results as means $\pm$ S.E.M. (with the number of determinations in parenthesis) and to use the students t test; p values were obtained from Fisher's tables[191]. Where the significance of a difference was in doubt and for all groups of results with 6 or fewer determinations, the Wilcoxon matched-pairs signed-ranks test was used[192] where possible.

CHAPTER III

RESULTS

A. DIAPHRAGM MUSCLE IN VITRO

The first two chapters are concerned with the choice of conditions for the incubations. In Chapter III the metabolism of glucose in diaphragm and liver was studied by measuring glucose-U-^{14}C incorporation into glycogen, oligosaccharides, intracellular glucose, CO_2 and lactate using autoradiochromatographic techniques[107]. The lack of a significant effect on parameters other than glucose-U-^{14}C incorporation into glycogen in diaphragm muscle, a confirmation of previous findings[107], led to the use of that parameter in the bioassays of insulin in Chapters V and VI. The possibility that previous diet can affect the metabolic activity of the isolated rat diaphragm suggested the experiment described in Chapter IV on the effects of controlled feeding on the basal incorporation of glucose-U-^{14}C into glycogen.

CHAPTER 1 : To determine the effect of Insulin, (10^{-2}U/ml), glucose and incubation medium volume on the rate of oxygen consumption by rat and lean mouse hemidiaphragm muscle in phosphate buffer.

These experiments were carried out prior to the experiments designed to test the sensitivity of mouse hemidiaphragm to insulin, in order to determine the optimal conditions necessary for those experiments.

Krebs-Ringer phosphate buffer, without any albumin, was used for all these experiments, the incubations being carrie out in Warburg vessels at 37°C.

RESULTS: Fig. 5 shows typical examples of the rate of oxygen uptake with time. It can be seen that the rat hemidiaphragm, approximately 80-120 mg. in weight, in 2 ml. of phosphate buffer,had a low and decreasing oxygen uptake with a medium pH of 6.8 after 100 minutes incubation. The same was true of mouse hemidiaphragm in 1 ml. incubation medium with or without glucose in the medium. The highest uptakes were obtained with mouse hemidiaphragms, usually weighing 20-35 mg. in 2 ml. of phosphate buffer; changing the glucose concentration from 0.2 % to 0.5 % made no difference to the rate of uptake. It can be seen that under these conditions the rate is approximately linear for at least three hours. Accordingly, for all experiments involving mouse hemidiaphragms or weights of diaphragm muscle less than 50 mg. at least 2 ml. of phosphate or bicarbonate buffer was used.

Gemmill[106] found the rate of oxygen uptake constant

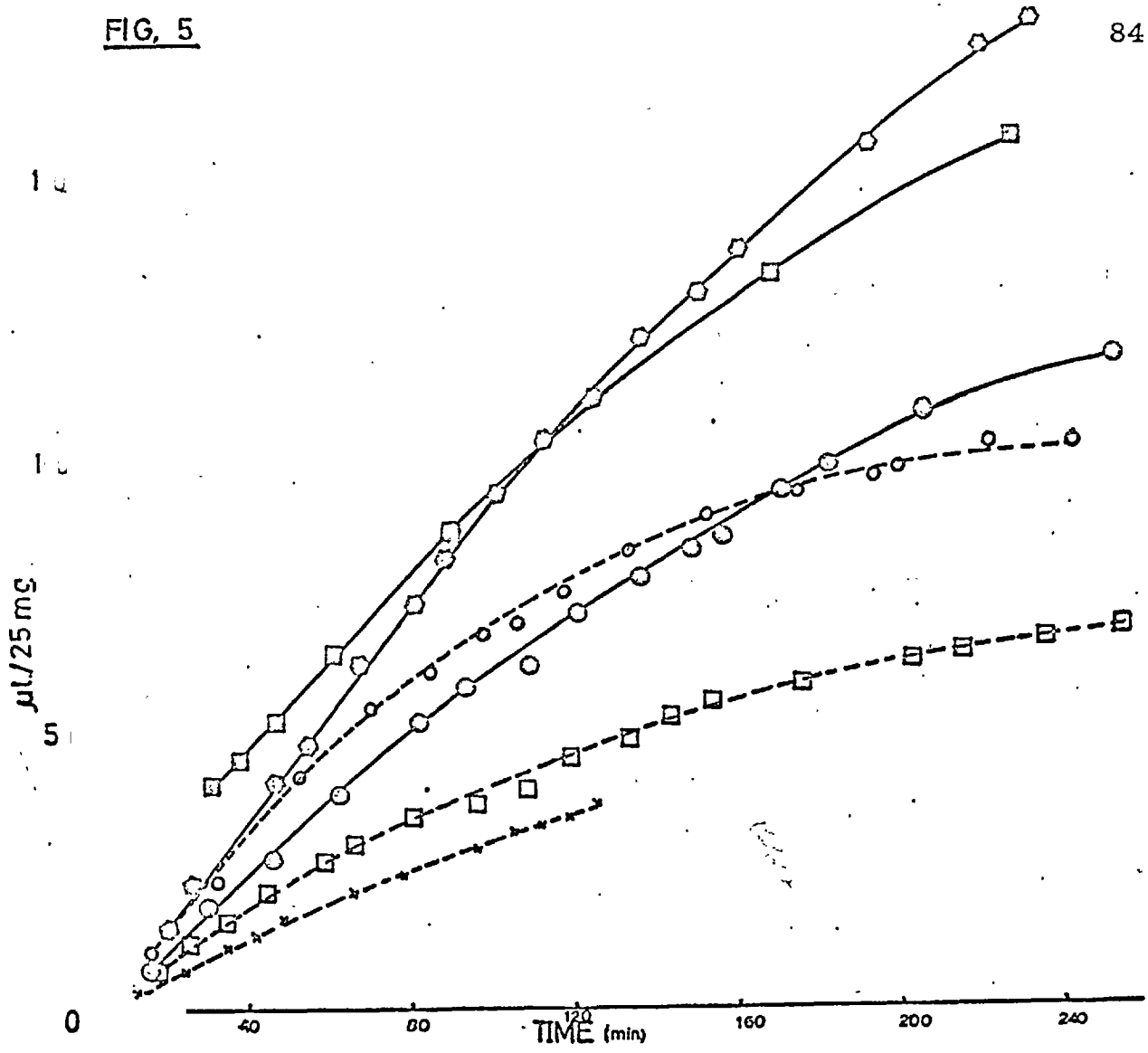


Fig. 5 : Rate of oxygen consumption in µl/25 mg. in hemi-diaphragms from lean rats and mice, incubated at 37°C in phosphate buffer equilibrated with O_2.

Other experimental details in text.

KEY

⬡	Mouse hemidiaphragm:	0.5%	medium glucose concentration :2ml. incubation volume
□	Mouse "	" "	glucose concentration :1ml. incubation volume
×	Rat "	"	glucose concentration :2ml. incubation volume
◘	Mouse "	0.2% "	glucose concentration :2ml. incubation volume
⊙	Mouse "	" "	glucose concentration :1ml. incubation volume
○	Fasted mouse "	No glucose	:1ml. incubation volume

for up to six hours in rat hemidiaphragms incubated in phosphate buffer, although the insulin effect on glycogen synthesis was maximal after 90-120 minutes[106,114]. For this reason, glucose incorporation into diaphragm glycogen was studied after 90 minutes under conditions which were found to give linear rates of oxygen uptake for at least 3 hours in mouse hemidiaphragms.

Insulin (10^{-2}U/ml.) had no effect on the uptake of oxygen by mouse hemidiaphragm muscle.

CHAPTER 2 : The effect of insulin (10^{-2}U/ml.) on the incorporation of glucose-U-^{14}C into glycogen in mouse hemidiaphragm muscle, incubated in phosphate and bicarbonate buffers.

The composition of the buffer is important in the study of the *in vitro* effects of hormones on isolated tissues[194,195]. These experiments were designed to see whether the presence of PO_4''' or HCO_3' was essential for the effect of insulin on the incorporation of glucose-U-^{14}C into glycogen in diaphragm muscle from the mouse. Earlier work[107] suggested that the response to insulin of this parameter was greater in bicarbonate buffer than in phosphate buffer. In Table 5, when the insulin effect, as an increment in incorporation, was calculated as the percentage of the value in the paired hemidiaphragm without insulin, a significant but very small difference between bicarbonate and phosphate buffers was only observed in diaphragm muscle from rats fed *ad libitum*. The

TABLE 5

The increment in glucose-U-^{14}C incorporation into total glycogen with insulin (10^{-2}U/ml.) as a percentage of the value obtained without insulin in mouse hemidiaphragms and rat quarterdiaphragms in phosphate and bicarbonate buffers.

Animal	Type of buffer	
	Krebs-Ringer phosphate	Krebs-Ringer bicarbonate
Rats fed ad lib.	$+9\pm3(6)$	$-3\pm3(8)$ ø
Rats fasted 24 hrs.	$+79\pm7(4)$ *	$+81\pm13(4)$ **
Mice fed ad lib.	$+4\pm10(4)$	$-21\pm12(4)$

(1) ** $p \leqslant 0.001$ 24 hr. fasted value signif. different from fed value in bicarbonate buffer.
(2) ø $p \leqslant 0.02$ signif. different from phosphate buffer.
(3) * $p \leqslant 0.001$ 24 hr. fasted value signif. different from fed value in phosphate buffer.

Mouse hemidiaphragms or rat quarterdiaphragms were incubated for 90 minutes at 37°C in 2ml. of buffer, pH 7.4, containing 0.2% glucose, no albumin and either water or 10^{-2}U/ml. crystalline ox insulin. The bicarbonate buffer was equilibrated with 95% O_2:5% CO_2 and the phosphate buffer with oxygen. The increment in glucose-U-^{14}C incorporation into glycogen, calculated as a percentage of the basal value is given as a mean $\pm$ S.E.M. with the number of determinations in parenthesis. Negative values were included in the results.

absence of an insulin effect in this group and in fed mice stressed the need for carrier albumin in the incubation medium in future experiments. In pilot experiments designed to test the reproducibility of measurements of glucose-U-^{14}C incorporation into glycogen, agreement was obtained on measurements from left and right hemidiaphragms from the same animal in both phosphate and bicarbonate buffers.

It was decided to use 0.2% dialysed albumin in the incubation medium on the basis of work done by other investigators[157] and Krebs-Ringer bicarbonate medium was chosen for all future experiments testing the insulin effect on glucose-U-^{14}C incorporation into glycogen in muscle on the grounds that it would be comparable to the much larger number of reports using this buffer in preference to the phosphate buffer, (especially with regard to work done in ob/ob mice). It was also felt that the reproducibility of the method for extracting glycogen was sufficiently good to warrant the use of this parameter as an index of insulin action.

CHAPTER 3 : The incorporation of glucose-U-^{14}C into "soluble" and "insoluble" glycogen and oligosaccharides, lactate and $^{14}CO_2$ in liver slices in bicarbonate buffer and in hemidiaphragm muscle in phosphate buffer from fed 6-8 month old ♂ lean and ob/ob mice with and without insulin (10^{-2}U/ml.) using autoradiochromatographic methods.

These experiments were undertaken to see if there was any difference in the qualitative pattern of radioactive metabolites isolated from obese and lean diaphragm and liver tissue incubated in the presence of glucose-U-^{14}C and to investigate the effect of insulin on these incorporations in the lean mouse tissues.

Plates 5 (a) and (b) show typical examples of the spots obtained after chromatographing diaphragm extracts from lean and ob/ob mice. The identity of the spots, taken from Beloff-Chain et al.[107] is given in Fig.6 and the quantitation of the spots by an automatic radiochromatographic scanner is given in Table 6. There was no major difference in the qualitative picture obtained on autoradiochromatography of diaphragms (Plates 5(a) & (b)) or of liver extracts from ob/ob and lean mice fed ad libitum. On any one day, and especially if paired extracts were spotted on the same sheet of paper for a monodimensional run, the differences between the insulin and the water extracts were always consisten in the quantitative results obtained for the spots comprising "soluble" glycogen and oligosaccharides.

Table 6 shows the actual values of incorporation of glucose-U-^{14}C into "soluble" glycogen and oligosaccharides, "insoluble" glycogen, "intracellular" glucose, lactate and

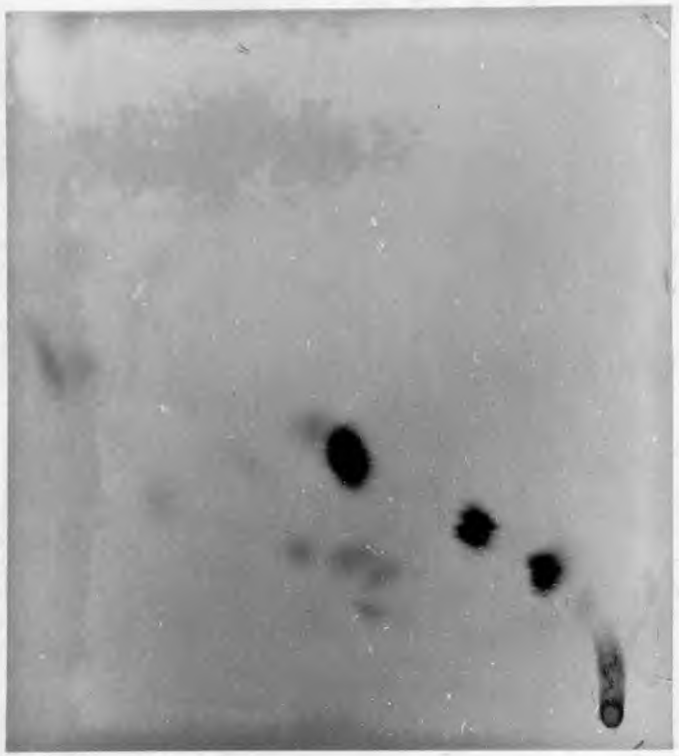


Plate 5 (a) & (b) : Bidimensional autoradiochromatograms of aqueous ethanolic extracts of mouse hemi-diaphragm muscle after incubation with glucose-U-^{14}C for 90 minutes at 37°C in the absence of insulin. (Experimental conditions in text).

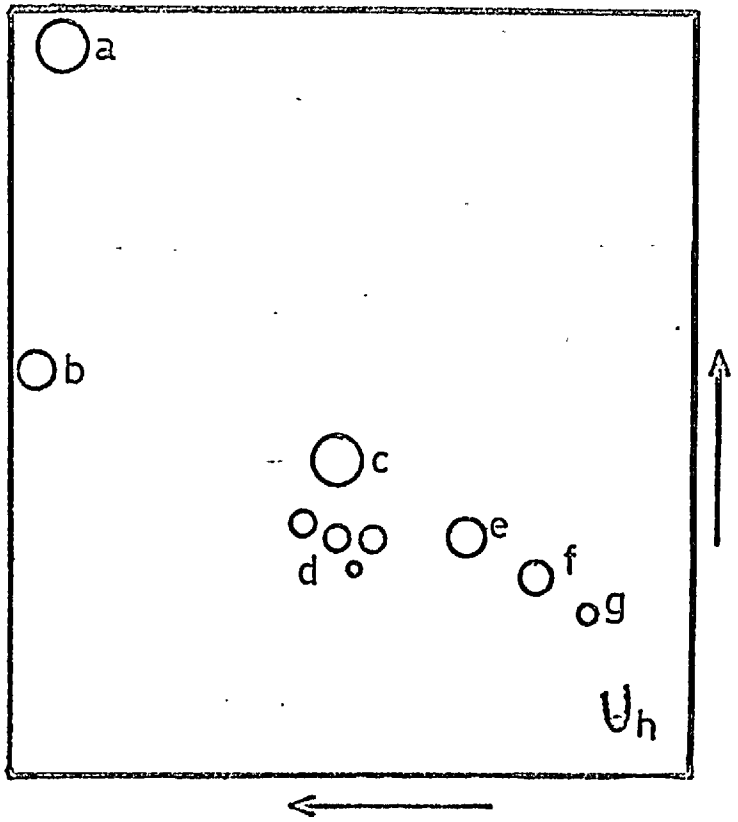
5(a) : ♂ lean mice, 6-8 months old fed *ad libitum*.

5(b) : ♂ *ob/ob* " " " " " " "

FIG. 6.

Key to the chromatograms in Plate 5(a)(b)

a = lactate
b = pyruvate
c = "intracellular" glucose
d = hexose phosphates
e = maltose
f = maltotriose
g = maltotetraose
h = "insoluble" glycogen



Arrows indicate direction of solvent flow.
Experimental details in the text.

TABLE 6

The effect of insulin (10^{-2}U/ml.) on the incorporation of glucose-U-^{14}C into "soluble" and "insoluble" glycogen (Method C), "intracellular" glucose, lactate and CO_2 in hemidiaphragm muscle from 6-8 month old ♂ lean mice fed ad libitum.

	Glucose-U-^{14}C incorporation in mμM/60 min./mg.		
	H_2O	+ Ins.10^{-2}U/ml.	Increment with ins.
"Soluble" glycogen & oligosaccharides	13.30±0.76 (8)	18.28±1.76* (8)	+4.86±1.85(8) (8)
"Insoluble" glycogen	0.65±0.17 (8)	0.59±0.10 (8)	-0.06±0.23 (8)
"Intracellular" glucose	3.95±0.58 (8)	4.26±0.39 (8)	+0.31±0.33 (8)
Lactate	12.88±0.79 (8)	15.08±1.03 (8)	+2.20±1.18 (8)
CO_2	8.87±0.72 (8)	7.95±0.45 (7)	-1.11±0.54 (7)

* significantly different from control $p \leqslant 0.05$

Hemidiaphragms were incubated with or without 10^{-2}U/ml. insulin in phosphate buffer, pH 7.4 containing 0.2% glucose and 1-5 μc glucose-U-^{14}C (sp. activity≏17μc/mg.) for 90 minutes under oxygen at 37°C. The number of determinations is given in parenthesis and is equal to the number of mice used. The results are presented in mμM/60 min./mg. wet weight, as the mean of all the determinations ± the standard error of the mean.

carbon dioxide in hemidiaphragms from lean mice, and the insulin effect here is presented as the increment in incorporation. Insulin at a concentration of 10^{-2}U/ml. had no effect on the incorporation of glucose-U-^{14}C into intracellular glucose, lactate or CO_2 and significantly affected only the incorporation into "soluble" glycogen and oligosaccharides, in agreement with earlier findings in the rat[107]. Insulin at the same concentration had no effect on any of these metabolites in liver slices incubated in vitro (Table 7).

Table 7, which shows the increment with insulin (10^{-2}U/ml.) in the incorporation of glucose-U-^{14}C calculated as a percentage of the basal value, also shows that the only insulin effect detected was on glucose-U-^{14}C incorporation into glycogen. When calculated as a percentage increment in paired hemidiaphragms, a significant effect of insulin on "insoluble" glycogen was evident, (which was not seen when expressed as the mean of the pooled incorporations in Table 6), as has also been found for the rat[107]. It can also be seen that there was no significant difference between the insulin effects on the incorporations into "soluble" glycogen and oligosaccharides, "insoluble" glycogen or on total glycogen (Method A: measured by prior digestion of the tissue in hot alkali) and no significant difference between the insulin effects on the incorporations into total glycogen (Method A) in hemidiaphragms from lean and ob/ob mice at 6-8 months of age, although the effect in ob/ob mice was noticeably smaller.

TABLE 7 : The increment in glucose-U-^{14}C incorporation effected by 10^{-2}U/ml. of insulin in total glycogen, "soluble" glycogen and oligosaccharides, "insoluble" glycogen, "intracellular" glucose, lactate and CO_2 expressed as a percentage of the control value in hemidiaphragm muscle and liver slices from lean and ob/ob mice.

TISSUE	DIAPHRAGM				LIVER
TYPE OF ANIMAL	Lean fed 2-4 mth.	Lean fed 6-8 mth.	ob/ob fed 6-8 mth.	Lean fasted 6-8 mth	Lean fed 6-8 mth.
Method A Total glycogen	+130±27(9)	+91±31(16)	+45±8(12)*	-	-
Method C "Soluble" glycogen & oligosaccharides	-	+58±28(9)	-3±24(4)	+51±49(5)	-18±8(12)
"Insoluble" glycogen	-	+71±46(10)	-	+29±48(5)	-28±10(11)
"Intracellular" glucose	-	+32±20(10)	-	+41±48(5)	+7±8(12)
Lactate	-	+10±11(10)	-	+23±15(5)	-3±13(12)
CO_2	-	+6±13(9)	-	-18±18(5)	-

* not significantly different from lean fed 6-8 month.

Experimental details for incubation of hemidiaphragms as for Table 6. Liver slices were incubated in bicarbonate buffer, pH 7.4 equilibrated with 95% O_2/5% CO_2 in otherwise similar conditions.

Values for glycogen and oligosaccharides using Method C have been derived using quantitative autoradiochromatographic techniques.

The number of determinations is given in parenthesis and is equal to the number of mice used. The results are presented as the mean of all the determinations ± the standard error of the mean. The insulin effects on incorporation into diaphragm total glycogen (Method A), "soluble" glycogen and oligosaccharides, and "insoluble" glycogen (Method C), and between different groups of lean mice were not significantly different.

CHAPTER 4 : The effect of two intragastric glucose loads (1 ml. 2M glucose) on the basal incorporation of glucose-U-^{14}C into glycogen in quarter-diaphragms from 24 hour fasted 4-9 month old ♂ mice

As ob/ob-RD mice have an artificial feeding pattern imposed on them and as there is no evidence that ob/ob mice have the same feeding habits as have been found in lean mice[196], it was possible that differences in glucose-U-^{14}C incorporation into glycogen could have been due to differences in feeding frequency. Diaphragms from fed animals, saturated with glycogen, would be expected to have a reduced incorporation of glucose-U-^{14}C into glycogen in vitro.

This hypothesis was tested by giving 24 hour fasted mice a known amount of glucose intragastrically. There were insufficient numbers of ob/ob mice available at the time of this experiment to determine whether the incorporation of glucose-U-^{14}C into diaphragm glycogen was different in ob/ob mice from that in lean mice when the fed state was thus artificially controlled ; (the blood glucose concentrations of young ob/ob and lean mice were not significantly different from each other after this intragastric glucose load). However, Table 8 shows the consistent decrease in incorporation of glucose-U-^{14}C into diaphragm glycogen after administration of a glucose load in a few lean mice, lean mice on a restricted diet and ob/ob-RD mice. Control mice all received an equal volume of saline.

Table 8 also shows that the values of incorporation of glucose-U-^{14}C into glycogen in fed mice given intragastric glucose loads were similar to those in fasted mice given glucose

TABLE 8

The effect of two intragastric glucose loads (1 ml. 2M glucose each) on the basal incorporation of glucose-U-^{14}C into glycogen in 24 hr. fasted 4-9 month ♂ mice.

Animal	+ saline	+ glucose
24 hr. fasted		
Lean	7.14±0.09(3) ①	3.73±0.23(8) ② *
Lean on restricted diet	8.11±0.62(12) ③	4.69±0.29(8) ② *
ob/ob-RD	7.79±0.85(8) ②	5.34±0.15 (7) ② **
Fed		
ob/ob-RD + single dose of ½ ml. Mglucose		4.48±0.36(4) ②
Lean " " " " " " "		3.05±0.50(4) ②
ob/ob " " " " " " "		2.61±0.53(6) ③

* significantly different from control $p \leq 0.001$
** " " " " $p \leq 0.02$

24 hour fasted mice were given two 1 ml. gastric intubations at 90 min. intervals of either 0.9% NaCl or 2M glucose and sacrificed 60-90 minutes later.

The quarterdiaphragms were incubated in bicarbonate buffer, pH 7.4, as described for the dose-response experiments (p.45). The incorporations of glucose-U-^{14}C into total glycogen in mμM/90min./mg.wet weight are presented as the means of the pooled determinations (in parenthesis) ± the S.E.M. The numbers of mice used are given in circles.

in the column above, indicating that the glucose loads given in 3 hours were sufficient to bring the fasted mice rapidly to a fed state. The results shown in Fig.7 (a) and (b) were also confirmed where, it can be seen that with larger numbers of mice, the basal incorporation of glucose-U-^{14}C to glycogen was higher in quarter-diaphragms from 24 hour fasted ob/ob mice than from fed ad libitum ob/ob mice at 2-4 months of age.

CHAPTER 5 : The effect of insulin on glucose-U-^{14}C incorporation into total glycogen in hemidiaphragms from fasted 12 month old ♂ ob/ob and lean mice *

These experiments on mouse hemidiaphragms differed from the experiments on mouse quarterdiaphragms reported in the next chapter in the incubation time which was 120 minutes, rather than 90 minutes, the older age of the mice, their fasted state, and the calcium chloride (aq.) concentration of the bicarbonate buffer which was 2.4 mM/L.

The results shown in Table 9 demonstrate a very clear response to insulin (10^{-2} U/ml) in hemidiaphragms from lean mice; the increment with insulin, expressed as a percentage of the incorporation of glucose-U-^{14}C into glycogen in the absence of insulin amounted to about 180%. The response of hemidiaphragm muscle from 12 month old ob/ob mice was very much reduced and amounted to no more than 20% of the glucose-U-^{14}C incorporation into glycogen in the absence of insulin. The basal glucose

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* In collaboration with Dr. C. Chlouverakis and P. White.

TABLE 9

The effect of insulin (10^{-2}U/ml.) on glucose-U-^{14}C incorporation into total glycogen in hemidiaphragm muscle from 24 hr. fasted ♂, ob/ob and lean mice at 12 months of age.

	Incorporation into total glycogen in dpm/120 min./mg. wet wt.x10^{-2}		
	Control	Ins.10^{-2}U/ml.	Increment with insulin
Lean	340±50(8)	953±104(8) *	+613±84(8)
ob/ob	443±54(8)	524±55(8)	+81 ±44(8)

* insulin value significantly different from control value $p \leqslant 0.001$

Hemidiaphragms from 24 hr. fasted ♂ mice were incubated at 37°C for 120 minutes in 2 ml. of bicarbonate buffer pH. 7.4 ($CaCl_2$ aq. 2.4 mM/L.) equilibrated with 95% O_2:5% CO_2 containing 0.2% albumin, 0.1% glucose and 0.2% albumin or 10^{-2}U/ml. insulin in 0.2% albumin. The glucose-U-^{14}C incorporation into glycogen, in dpm/120 min./mg. is expressed as the mean of the pooled determinations ± the S.E.M., with the number of determinations, equal to the number of mice used, in parenthesis.

incorporation into glycogen in the absence of insulin can be seen to be slightly but not significantly higher than the values from ob/ob hemidiaphragms. Although the response to insulin shown in Table 9 was highly significant, it was clear that there was considerable variation shown in the values for the incorporation of glucose-U-^{14}C into glycogen by different hemidiaphragms. Further experiments not presented here showed that, using this method, clear cut differences could not be shown statistically unless large numbers of animals were used, because of a small but significant number of non-responding diaphragms[115]. To rectify this, two modifications were introduced to improve the protocol: the use of quarterdiaphragms instead of hemidiaphragms enabled more than one concentration of insulin to be tested and thus made possible sufficient determinations for a dose-response curve, and secondly, the quarterdiaphragms were not randomly pooled as were the hemidiaphragms in this chapter - the four quarterdiaphragms coming from one animal were distributed to one control flask and three flasks each containing different concentrations of insulin so that all determinations were paired. It was also decided to study the mice in the fed state as it was felt that the fasted state might mask a lack of response present in the fed state, under which conditions an insensitivity to insulin would be physiologically significant.

CHAPTER 6 : The response and sensitivity towards insulin of quarterdiaphragms from fed lean and obese mice, (ob/ob) and from obese mice on a restricted diet (ob/ob-RD) at 2-4 and 6-8 months of age in bicarbonate buffer containing 0.1% glucose

The results given in Fig. 7 (a) and (b) show the incorporation of glucose-U-^{14}C into glycogen in the diaphragm muscle from mice at 2-4 months of age and 6-8 months of age respectively, in the absence of insulin and in the presence of 5×10^{-3}U/ml. of insulin. Results with 5×10^{-4}U/ml. insulin are shown separately in Table 10 so as to simplify the presentation in Fig.7 (a) and (b).

These results show that in the 2-4 months age group there appeared to be a greater incorporation of glucose into glycogen in diaphragms from lean mice or ob/ob mice fasted for 24 hours than those obtained in ob/ob fed ad libitum. There was no significant insulin effect in ob/ob mice in this age group, either fed or fasted, whereas diaphragms from lean mice showed a clear response to insulin at both 5×10^{-4}U/ml. and 5×10^{-3}U/ml. concentrations. Obese mice on a restricted diet (ob/ob-RD) were not investigated at this age.

In the 6-8 months age group the diaphragms from ob/ob-RD mice had a lower incorporation of glucose-U-^{14}C into glycogen than those observed with lean mice or ob/ob mice diaphragms, but this was restored to normal by insulin at 5×10^{-4}U/ml. and 5×10^{-3}U/ml. Diaphragms from lean mice also responded to insulin at both these concentrations but there was no significant effect at any insulin concentration tested in diaphragms from ob/ob mice fed ad libitum at 6-8 months of age.

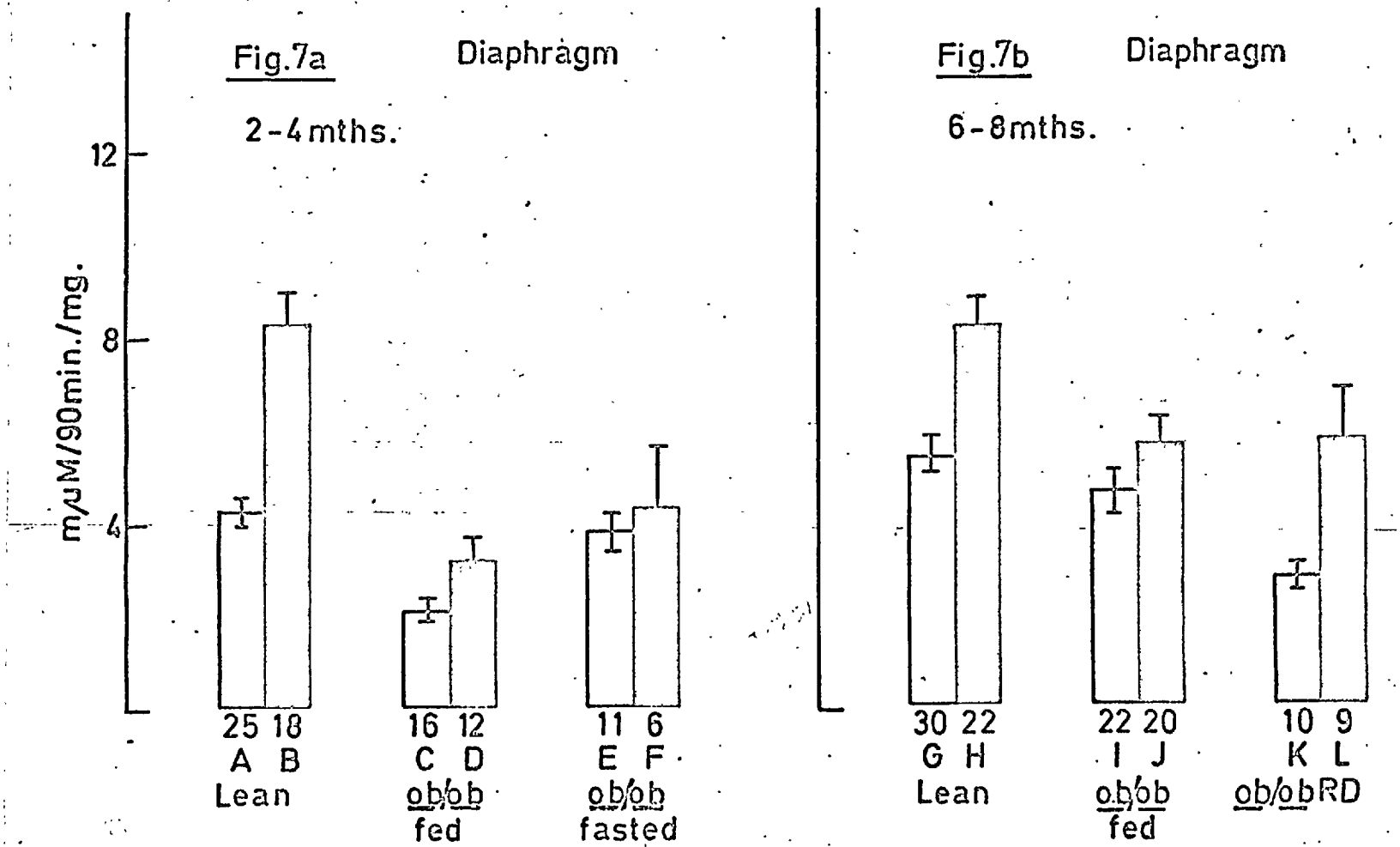


Fig. 7(a)(b): Legend on p.101

LEGEND FOR Fig. 7 (a) (b)

The incorporation of glucose-U-^{14}C into glycogen in diaphragm muscle from fed lean and obese (ob/ob) mice and from fasted obese mice and from fed obese mice on a restricted diet (ob/ob-RD) at 2-4 months and 6-8 months of age, with and without insulin.

Diaphragms incubated in bicarbonate buffer, pH 7.4 containing 1.2 mM/l of Ca^{++}, 0.2% dialysed albumin, 0.1% glucose and 1-5μc glucose-U-^{14}C (sp. activity ≏ 17μc/mg.), for 90 minutes at 37° C in an atmosphere of 95% O_2/5% CO_2.

The number of determinations is given below each column and in most cases came from the same number of mice (6 fasted ob/ob mice used). The standard error of the mean of the observations is shown as a bar above each column.

White columns - without insulin
Black columns - 5×10^{-3} U/ml. crystalline ox insulin in 0.2% dialysed albumin solution

Significant differences: $p \leq 0.001$ - AB, AC, CE, GH, GK.
$p \leq 0.01$ - KL.

All other comparisons not significant.

TABLE 10

The incorporation of glucose-U-^{14}C into glycogen in quarter-diaphragms from fed lean and obese mice, and obese mice on a restricted diet (ob/ob-RD) at 2-4 months and 6-8 months of age with and without 5 x 10^{-4}U/ml. of insulin.

Glucose-U-^{14}C incorporation into total glycogen mμM/90 min./mg.			
2-4 months of age			
	Lean	ob/ob fed	ob/ob fasted
Control	4.24±0.30(25)	2.10±0.26(16)*	3.81±0.38(11)x
Insulin 5 x 10^{-4}U/ml.	7.00±0.80(18)ø	3.30±0.61(12)	4.10±1.07(5)
6-8 months of age			
	Lean	ob/ob fed	ob/ob-RD
Control	5.39±0.39(30)	4.56±0.48(22)	2.76±0.25(10)*
Insulin 5 x 10^{-4} U/ml.	7.14±0.62(17)øø	4.81±0.41(19)	4.72±0.87(9)øø

* $p \leq 0.001$ control significantly different from lean control.

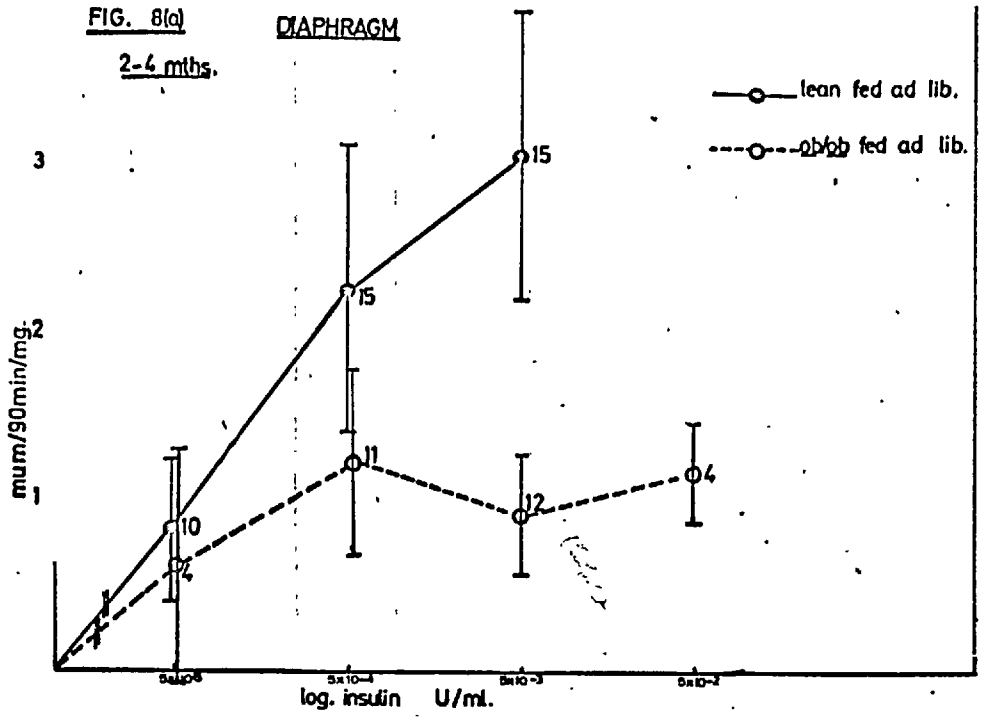
ø $p \leq 0.001$ insulin value significantly different from control.

øø $p \leq 0.05$ " " " " " "

x $p \leq 0.001$ fasted value significantly different from fed value.

Quarterdiaphragms were incubated in bicarbonate buffer for 90 minutes at 37°C. Conditions were identical to those given in Fig.7(a)(b)except for the lower insulin concentration used here, viz. 5 x 10^{-4}U/ml. The incorporation of glucose-U-^{14}C into total glycogen, in mμM/90 min./mg. is given as the mean of all the determinations ± the S.E.M. with the number of determinations in parenthesis. Only 6 24hr.-fasted ob/ob mice were used; in most other cases the number of animals sacrificed was equivalent to the number of determinations made.

It should be noted that these results are calculated as the means of all the individual incorporations obtained under the given experimental conditions; the number of determinations is indicated in the figures. In Fig.8 (a) and (b) the results of the same experiments, shown in graphical form, are expressed as a mean of the insulin effect determined as an increment in glucose-U-^{14}C incorporation into glycogen. These values were obtained from paired experiments, in which parts of the same diaphragm were incubated without insulin and parts with insulin. The results of experiments with four insulin concentrations, 5×10^{-5}U/ml., 5×10^{-4}U/ml., 5×10^{-3}U/ml. and 5×10^{-2}U/ml., are given. Fig. 9 (a)(b) shows graphs of the same results but the insulin effect as the increment in incorporation of glucose-U-^{14}C into glycogen is here presented as a percentage of the basal incorporation without insulin. An index of the accuracy of the bioassay of insulin using the incorporation of glucose-U-^{14}C into glycogen by mouse quarterdiaphragms <u>in vitro</u> was obtained by dividing the standard deviation of the highest increment by the highest increment in incorporation per $\log_{10}$ increase in insulin concentration (viz. the slope where it is steepest). This calculation gave approximate values for "λ" of 1.34 and 0.88 for 6-8 month and 2-4 month old mice using pooled values and 0.60 and 1.46 respectively using increments of glucose-U-^{14}C incorporation into total glycogen. Wardlaw[143] quotes a λ of 0.45 (measuring glycogen synthesis in mouse hemidiaphragms) and Oyama[142] a λ of 0.59 (measuring glucose uptake in mouse hemidiaphragms). Thus the accuracy of this bioassay is somewhat less than those reported by these authors and can be attributed to the use of the less suitable quarter-



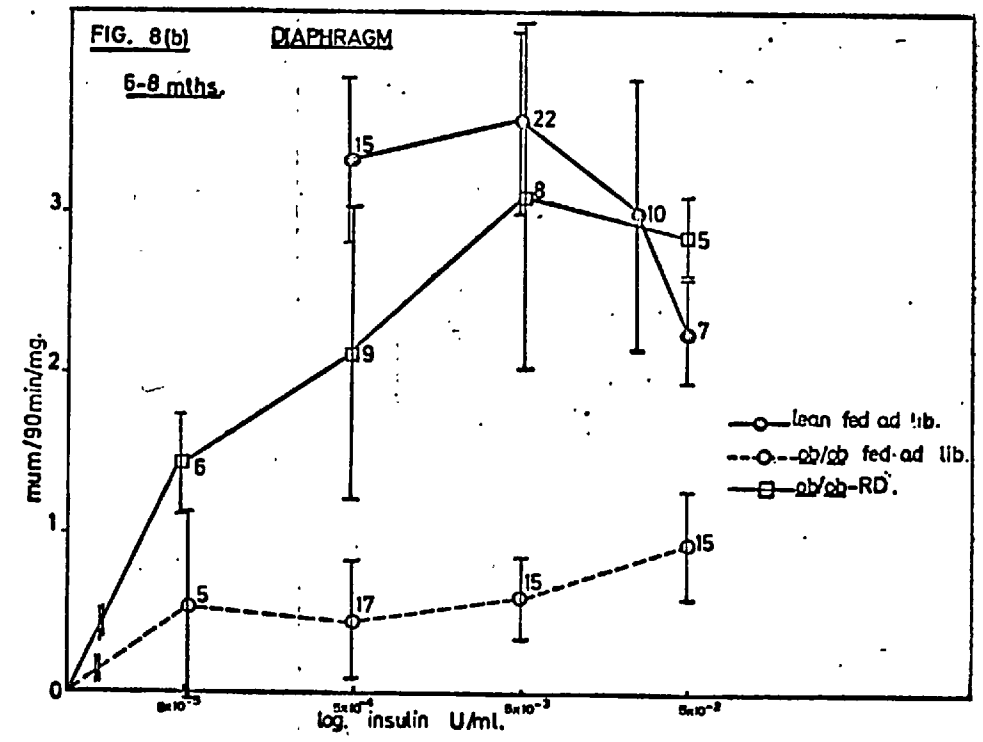
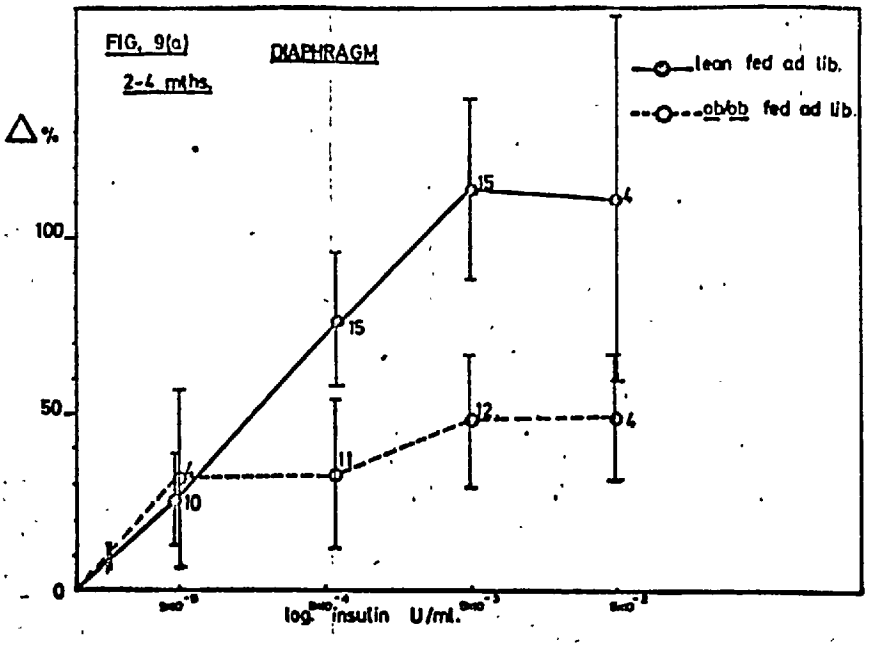


Fig.8 (a) (b) : Experimental details as for Fig.7 (a) (b). The insulin effect is expressed as the increment in glucose-U-^{14}C incorporation into glycogen in mμM/90 Min./mg. The number of determinations is given beside each point.



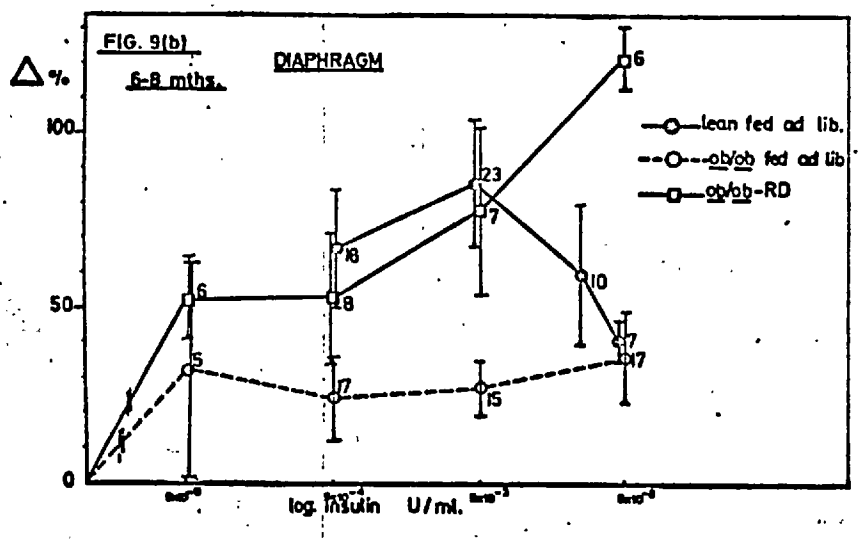


Fig.9 (a)(b) : Experimental details as for Fig.7(a)(b).

The insulin effect is expressed as the increment in glucose-U-^{14}C incorporation into glycogen as a percentage of the basal value. The number of determinations is given beside each point.

diaphragm preparation. However, this value of λ compared favourably with the λ obtained for the bioassay of insulin using mouse epididymal fat pads (p.119). The considerable variation in the responses of quarterdiaphragms from different animals (common to all hormone bioassays) was shown by the large S.E.M. values which, when there was no response to insulin, were larger than the means from which they were calculated because negative values were also included.

It can be clearly seen from these results that diaphragms from lean mice in both age groups responded significantly to insulin at all concentrations tested, and there was no significant change in the response to insulin between the two ages studied. The surprising decrease in response between 5×10^{-3}U/ml. and 5×10^{-2}U/ml. is difficult to explain and was not seen in adipose tissue dose response curves. In the 6-8 months age group, diaphragms from ob/ob mice showed a significantly smaller insulin effect with all concentrations of insulin whether the effect was expressed in terms of the actual increment (Fig. 8 (a)(b)) or the increment as a percentage of the basal value (Fig.9 (a)(b)). The insulin effect on the diaphragms of obese mice on a restricted diet (ob/ob-RD) at 6-8 months of age was normal with a concentration of 5×10^{-3}U/ml. of insulin.

In the 2-4 months age group the reduced insulin effect seen in the ob/ob mice of the older age group was not so evident. As the basal incorporation of glucose into glycogen was reduced in the 2-4 months age group (Fig. 7 (a)) the insulin effect, when calculated as a percentage increase of the basal incorporation value, showed no significant difference

between ob/ob and lean diaphragm muscles at either 5×10^{-4} U/ml. or 5×10^{-3} U/ml. insulin. Expressed simply as an increment, the insulin effect of diaphragms of fed ob/ob mice was significantly different from that of diaphragms of lean mice only at the higher insulin concentrations (Fig. 8(a)).

B. EPIDIDYMAL FAT PADS IN VITRO

Ever since its inception as an experimental preparation[105,128] for *in vitro* studies on the metabolism of adipose tissue, the rat epididymal fat pad has been exploited by many investigators[65,89ab,90,94,96,101,102,119,122-127] who studied the response of this tissue to various hormones and in particular to insulin. The effect of insulin on glucose-1-^{14}C oxidation has been used here (except for the experiment on p.109) in a bioassay adapted from that reported for rat epididymal adipose tissue by Martin et al.[125-127].

It has been shown that the sensitivity of rat epididymal fat pads to insulin was independent of the glucose concentration in the medium over a wide range[94]. 0.1% glucose was chosen because it was a reasonable approximation to the blood glucose concentration of lean mice.

Omental adipose tissue was used when female mice were studied (Chapter B7) in order to avoid any possible variations in response with the menstrual cycle in parametrial adipose tissue from these mice.

CHAPTER 7 : To determine the effect of insulin (10^{-3}U/ml.) on glucose-U-^{14}C incorporation into lipid in omental adipose tissue from fed 12 month old ♀ ob/ob and lean mice *

Incubations were carried out for 120 minutes at 37°C in 2ml Krebs-Ringer bicarbonate solution (with 2.4mM/L aq. $CaCl_2$), pH7.4 containing 0.2% crystalline undialysed bovine albumin Fr.V. and 0.1% glucose, and equilibrated with 95%O2:5%CO2. It should be noted that these conditions differ from those used in Chapters B8, B9, B10.

Table 11 shows a clear response to 10^{-3}U/ml. of insulin in tissue from lean mice and no significant response in tissue from ob/ob mice. The basal incorporation of glucose-U-^{14}C into lipid in the adipose tissue from ob/ob mice was 30% of that found in tissue from lean mice.

CHAPTER 8 : The response and sensitivity towards insulin of epididymal fat pads from lean, obese (ob/ob) and obese mice on a restricted diet (ob/ob-RD) at 2-4 and 6-8 months of age

The results shown in Fig.10 (a) and (b) show the effect of 10^{-4}U/ml. insulin on the oxidation of glucose-1-^{14}C to $^{14}CO_2$ per mg. wet weight of epididymal fat pads taken from the same groups of animals used in the diaphragm muscle studies (Chapter A6). These results were also calculated as a mean of all the values obtained under the different experimental conditions. Results with 5×10^{-5}U/ml. insulin are shown separately in Table 12 so as to simplify the presentation in Fig. 10 (a) and (b).

* In collaboration with Dr. C. Chlouverakis and P. White.

TABLE 11

The effect of insulin (10^{-3}U/ml.) on glucose-U-^{14}C incorporation into lipid in omental adipose tissue of ♀ fed ob/ob and lean mice at 12 months of age.

	Incorporation of glucose-U-^{14}C into lipid dpm/mg./120 min.		
	Control	Ins. 10^{-3}U/ml.	Increment with insulin
Lean	314±36(8)	1047±160(8)*	+732±140(8)
ob/ob	109±10(8)	134±21(8)	+25±15(8)

* insulin value significantly different from control value $p \ll 0.001$

Experimental details in text. The results are given as the mean of the determinations ± the S.E.M. with the number of determinations, equal to the number of mice used, in parenthesis.

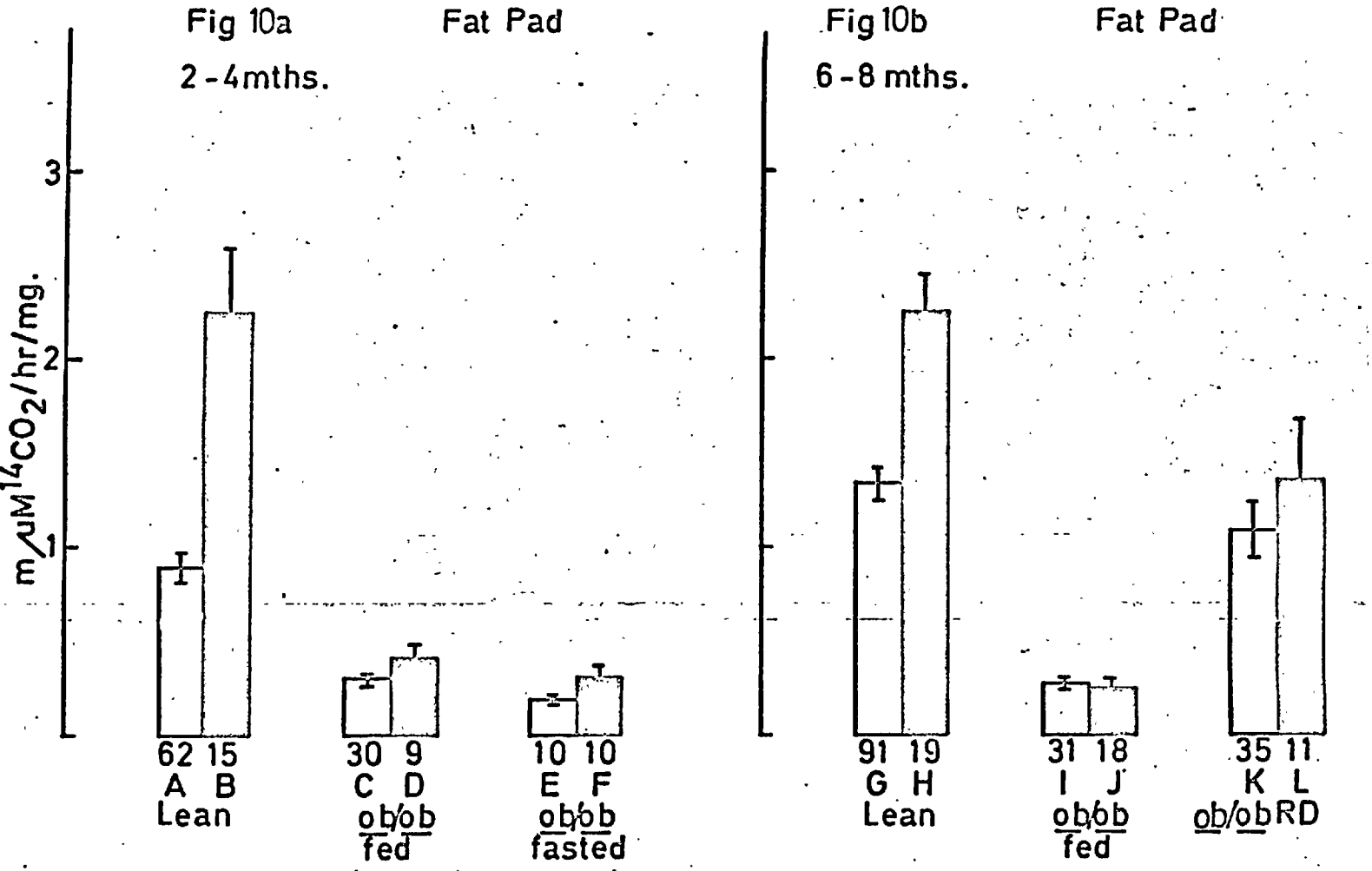


Fig. 10(a)(b): Legend on p.112.

LEGEND TO FIG. 10(a)(b)

The oxidation of glucose-1-^{14}C to $^{14}CO_2$ by epididymal fat pads from fed lean and obese (ob/ob) mice and from fasted obese mice and from fed obese mice on a restricted diet (ob/ob-RD) at 2-4 months and 6-8 months of age, with and without insulin.

Fat pads incubated in bicarbonate buffer, pH7.4, containing 1.2mM/L of Ca^{++}, 4% dialysed albumin, 0.1% glucose and 1-2 µc glucose-1-^{14}C (sp. activity ≏ 1.60 µc/mg.), for 2 hours at 37°C in 95%O_2/5%CO_2.

The number of determinations is given below each column. At 2-4 months of age, 59 lean, 10 fed obese and 6 twenty-four hour fasted obese mice were used, while in the 6-8 month age group, 85 lean, 21 obese and 12 ob/ob-RD mice were used.

White columns - without insulin

Black columns - 5×10^{-4} U/ml. crystalline ox insulin in 0.2% dialysed albumin solution.

Significant differences: $p \leqslant 0.001$ - AB, AC, AE, AG, GH, GI, IK.

All other comparisons not significant.

LEGEND TO TABLE 12

Conditions are identical to those described in Fig.10(a)(b). The oxidation of glucose-1-^{14}C to $^{14}CO_2$ in mµM/60 min./mg. is given as the mean of all the determinations ± the S.E.M. with the number of determinations in brackets. Only 6 24-hour fasted ob/ob mice were used; in most other cases the number of animals sacrificed was equivalent to the number of determinations made.

TABLE 12

The oxidation of glucose-1-^{14}C to $^{14}CO_2$ in epididymal fat pads from lean and obese mice, and obese mice on a restricted diet (ob/ob-RD) at 2-4 months and 6-8 months of age with and without insulin.

2-4 months of age

	Lean	ob/ob fed	ob/ob fasted 24 hrs.
Control	0.89±0.08(62)	0.29±0.03(30)+++	0.19±0.02(10)‡++
Insulin concentration			
5 x 10^{-5}U/ml.	1.21±0.15(13)	0.37±0.05(10)	0.26±0.06(10)
5 x 10^{-4}U/ml.	2.24±0.35(15)***	0.41±0.07(9)	0.31±0.05(10)
5 x 10^{-3}U/ml.	-	0.44±0.08(10)*	-

6-8 months of age

	Lean	ob/ob fed	ob/ob-RD fed
Control	1.33±0.08(91)øøø	0.26±0.03(32)+++	1.08±0.15(35)++
Insulin concentration			
5 x 10^{-5}U/ml.	1.95±0.25(20)**	0.28±0.05(20)	1.13±0.19(12)
5 x 10^{-4}U/ml.	2.24±0.21(19)***	0.24±0.04(18)	1.35±0.32(11)
5 x 10^{-3}U/ml.		0.31±0.04(21)	1.38±0.21(12)

*** $p \leq 0.001$ significantly different from control values.
** $p \leq 0.01$ " " " " "
* $p \leq 0.05$ " " " " "
++ $p \leq 0.001$ " " " ob/ob fed ad lib. values.
+++ $p \leq 0.001$ " " " lean values.
øøø $p \leq 0.001$ " " " value at 2-4 mths.of age.
+ $p \leq 0.05$ " " " ob/ob fed ad lib. values.

LEGEND ON p.112

These results show that glucose-1-^{14}C oxidation was considerably higher in the tissue from the lean mice than in that of fed or fasted ob/ob mice in both age groups ($p < 0.001$) and this oxidation increased with age in lean mice ($p < 0.001$). Fat pads from lean mice responded significantly to insulin at a concentration of 5×10^{-4} U/ml. and above in the 2-4 month age group ($p < 0.001$) and at 5×10^{-5} U/ml. and above in the 6-8 month age group ($p < 0.01$), but there was no significant insulin effect on glucose oxidation at these concentrations in the fat pads from 2-4 month or 6-8 month old ob/ob mice whether fasted or fed. A significant insulin effect was obtained only in the 2-4 month old ob/ob group with an insulin concentration of 5×10^{-3} U/ml. ($p < 0.05$). At 6-8 months of age, the fat pads from the ob/ob-RD mice were not significantly responsive to insulin even at 5×10^{-3} U/ml. although the normal basal values (i.e. without the addition of insulin) did not differ significantly from those of lean mice. The basal oxidation of glucose-1-^{14}C in pads from 24 hour fasted ob/ob mice was significantly less than that in pads from lean ($p < 0.001$) or ob/ob mice ($p < 0.05$) fed ad libitum in the 2-4 month age group.

In Fig. 11 (a) and (b) the results are expressed as an increment in glucose oxidation in the presence of varying insulin concentrations; the values were obtained from paired experiments in which epididymal fat pads from the same animal were incubated without and with insulin at the concentrations indicated on the figure. Fig.12 (a) and (b) show graphs of the same results but the insulin effect as the increment in oxidatio of glucose-1-^{14}C to $^{14}CO_2$ is here presented as a percentage of the basal incorporation value (i.e. without insulin). The index

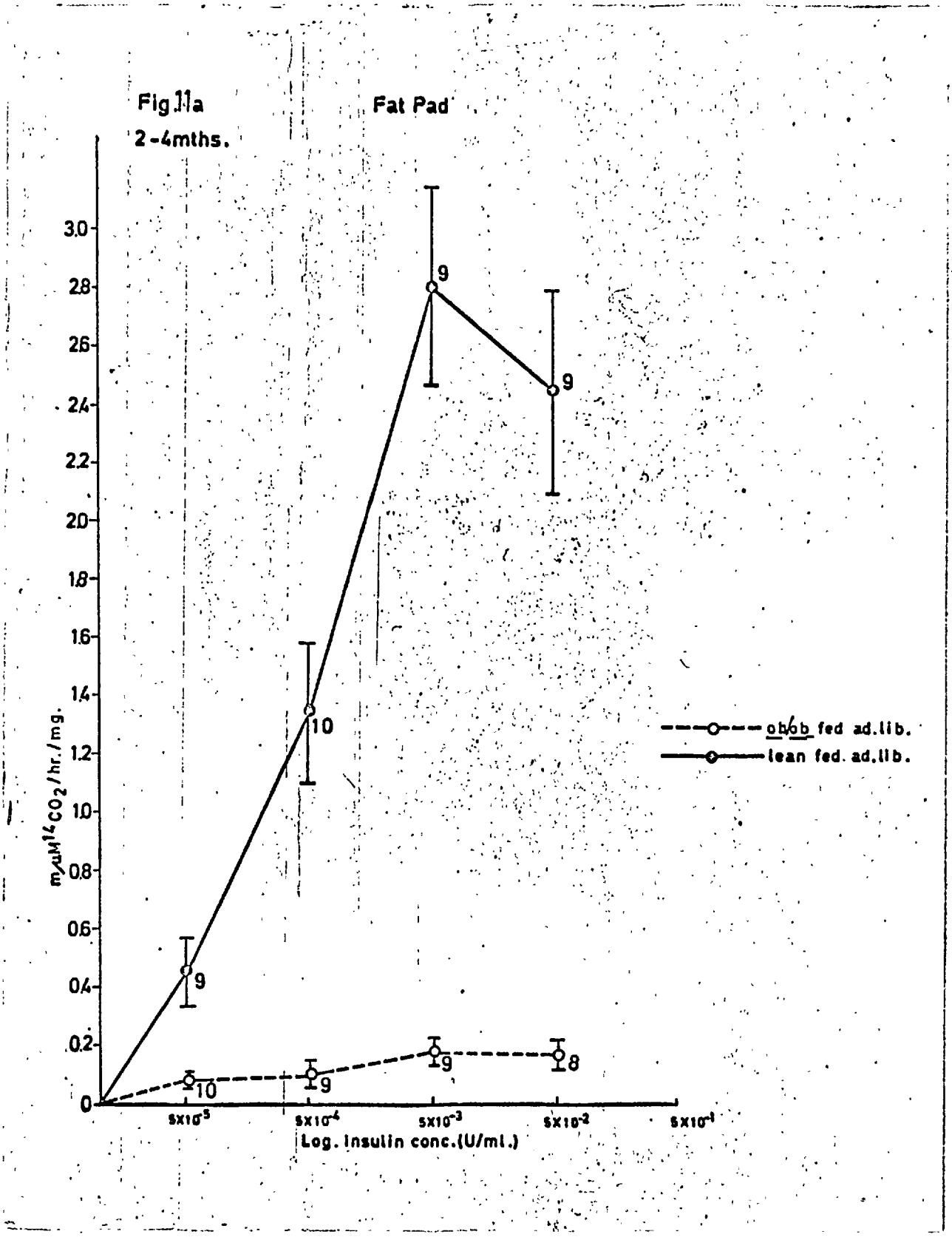


Fig. 11(a): Legend on p. 117

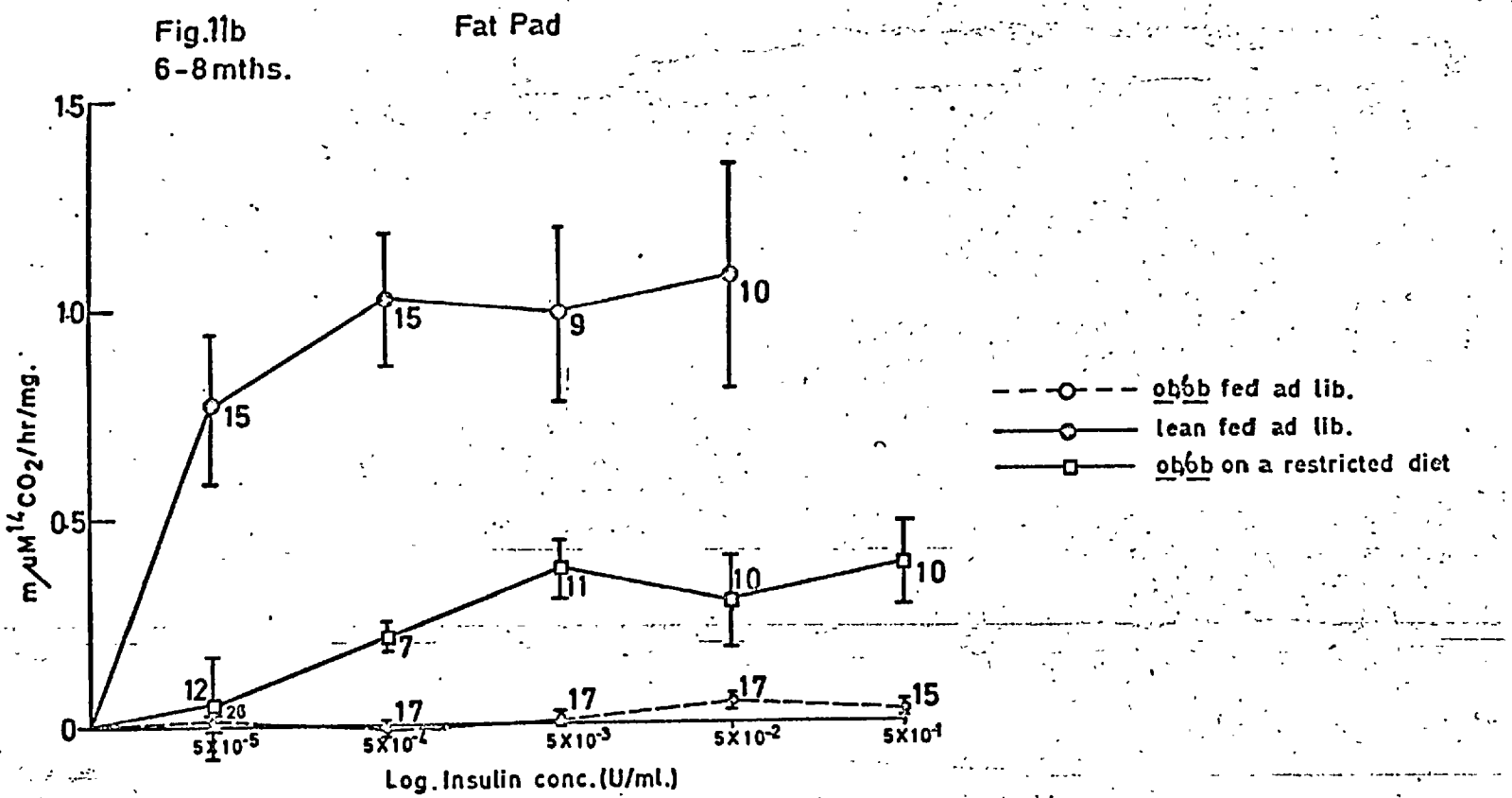


Fig. 11(b): Legend on p.117.

LEGEND TO FIG. 11 (a)(b)

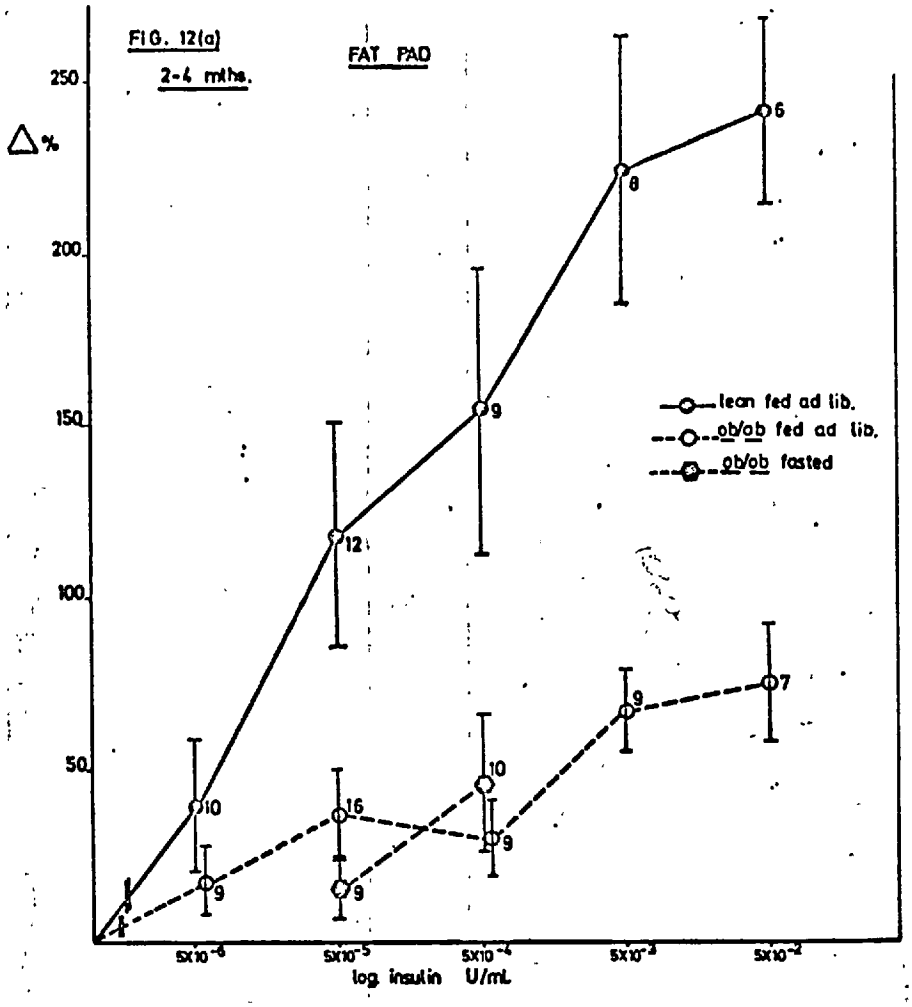
The increment in the oxidation of glucose-1-^{14}C to $^{14}CO_2$, effected by different concentrations of insulin in the epididymal fat pads from fed lean and obese (ob/ob) mice and from fed obese mice on a restricted diet (ob/ob-RD) at 2-4 and 6-8 months of age.

Incubation conditions as in Fig. 10(a)(b).

The number of paired determinations, obtained from the same number of mice, is given beside each point. The standard error of the mean of the observations is shown as a bar beside each point. The ordinate was calculated as the difference between the value with insulin and the value without insulin from pads from the same animal.

In Fig. 11(a) $p \leqslant 0.001$ - All points on each of the two curves are significantly different from points on the other curve.

In Fig. 11(b) $p \leqslant 0.001$ - All points on each of the three curves are significantly different from points on the other curves except at 5×10^{-5} U/ml. insulin where the ob/ob-RD value is not significantly different from the ob/ob value.



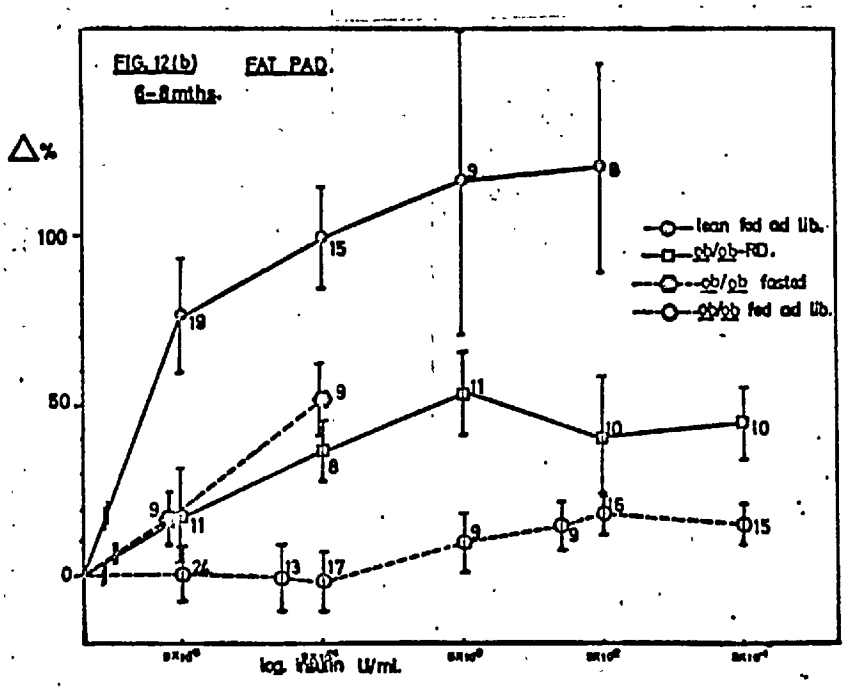


Fig.12 (a)(b) : Experimental details as for Fig.10(a)(b). The insulin effect is expressed as the increment in glucose-1-^{14}C oxidation to $^{14}CO_2$ as a percentage of the basal value. The number of determinations is given beside each point.

of precision, "λ", of the in vitro bioassay (see p. 103) using epididymal adipose tissue, was calculated to be 0.61 and 1.03 in 2-4 month and 6-8 month old lean mice using the increment in glucose-1-^{14}C oxidation to $^{14}CO_2$ as a criterion of the insulin effect. This compares with 1.46 and 0.60 for the data using diaphragm tissue and 0.25 for isolated adipocytes.

From Fig. 11 (a) and (b) it is evident that tissue from fed ob/ob mice at both ages tested was completely unresponsive to insulin at all concentrations examined, including a concentration as high as 5×10^{-1}U/ml. However, when the insulin effect was expressed as a percentage of the basal value, ob/ob mice had pads that, in keeping with the behaviour of pads from young lean mice, were more responsive at the younger age group, responding with an insulin effect of +75% with 5×10^{-2}U/ml. insulin. The fat pads from lean mice appeared to have a more pronounced insulin effect at concentrations above 5×10^{-3}U/ml. in the 2-4 month age group than in the 6-8 month old animals ($p < 0.01$) (Fig. 11(a)(b)); in fact pads from young mice were as responsive at 5×10^{-5}U/ml. insulin as pads from older mice were at 5×10^{-3}U/ml. - an increase in sensitivity with age of 100-fold. In 6-8 month old lean mice a maximum effect was obtained at 5×10^{-5}U/ml. whereas in the 2-4 month age group the insulin effect continued to increase up to 5×10^{-3}U/ml. and this effect was nearly two to three times greater than that at the older age group, (Fig.11 (a) and (b) and Fig.12 (a) and (b)). In fact, this response was nearly 250% of the basal value without insulin and was

considerably more than the highest effects obtained with isolated adipocytes. There was a significant insulin effect in the ob/ob-RD group at 6-8 months of age at concentrations of insulin of 5×10^{-4}U/ml. and over, although the effect was significantly less than that obtained with the lean animals; the minimum concentration required to give a significant insulin effect was 5×10^{-4}U/ml. (compared with a concentration of 5×10^{-5}U/ml. insulin for pads from lean mice) and the concentration at which the insulin effect was maximal was 5×10^{-3}U/ml, (compared to a concentration of 5×10^{-4}U/ml. of insulin for pads from lean mice).

Epididymal fat pads from 24 hour fasted ob/ob mice 2-4 months old were also relatively unresponsive to insulin when compared with lean mice (Fig.12(a)). The insulin effect in pads from fasted ob/ob mice 6-8 months old was not significantly different from that of pads from fed ob/ob-RD mice 6-8 months old at 5×10^{-5} U/ml.or 5×10^{-4}U/ml. of insulin (Fig. 12(b)).

C. ADIPOCYTES IN VITRO

The preparation of isolated fat cells has been modified from that published by Rodbell[136] for use with rat adipose tissue in the use of purified collagenase (see p.57), the use of albumin-free buffer for the incubation with the collagenase, and the shortened time needed for this incubation (usually 30 minutes). The reduction in the time needed for the digestion of the adipose tissue with pure collagenase was not because of any greater potency evident with use of the pure enzyme but because it was felt desirable to keep the amount of time spent on the preparative procedure down to a minimum as prolonged incubation with crude collagenase is known to damage the adipocytes' response to insulin[93]. The filtering of the tissue fragments through a nylon mesh with pore size of 250μ was essential if the incubation time with collagenase was to be reduced but may have excluded many "giant" adipocytes from the suspensions from ob/ob mice and may have contributed to lysis of the cell suspension and loss of the response to insulin (which has been shown to occur if adipocytes are not prepared with care[93]).

In these experiments, no quantitative data is available for the basal glucose oxidation (in the absence of insulin) in adipocytes from ob/ob or lean mice, as were given in the epididymal fat pad experiments. This was because of the technical difficulties involved in obtaining a reliable index of the number of intact cells present on different days in different cell suspensions from lean and ob/ob mice. It appeared that the fragility of the cells prepared from the adipose tissue of ob/ob mice was greater than in those prepared

from lean mice as indicated by the presence of more fat droplets floating on the adipocyte suspension during the preparation of the cells and by the absence of the large adipocytes present in the intact pads from ob/ob mice (Fig. 16). Therefore, the insulin effect has been measured for each separate cell suspension, and calculated as the percentage increase of the glucose-1-^{14}C oxidized in the absence of insulin. Each dose-response curve was obtained from a single cell suspension prepared with tissue from 6 rats, 1-3 ob/ob or ob/ob-RD mice, or 15-20 lean mice. The response at each insulin concentration was obtained by taking the standard error of the difference of the means of 3-4 determinations, with and without insulin.

The mean λ for the dose-response curves of lean and ob/ob adipocytes at 2-4 months of age was 0.27 for 5 suspensions, and at 6-8 months of age, was 0.24 for 12 suspensions.

CHAPTER 9 : To test the effect of insulin on rat adipocytes prepared with chromatographically purified collagenase

These experiments were initiated in order to establish that the use of the pure collagenase enzyme did not drastically alter the sensitivity and response to insulin of rat adipocytes.

Using bicarbonate buffer at pH7.4, equilibrated with 95% O_2 : 5% CO_2 containing 0.2% glucose and 4% crystalline dialysed albumin, rat adipocytes (about 80 μN per aliquot) were incubated without and with 10^{-4} and 10^{-3} U/ml. of insulin. The results of five experiments are shown in Table 13(a). It is clear that the effects, although variable on different days and using rats of no particular weight range, were still marked despite the high medium glucose concentration of 0.2% and the absence of albumin as fatty acid acceptor in two of the experiments. It was concluded that the use of the purified collagenase was suitable for studies on the response and sensitivity of isolated adipocytes to insulin.

However, experiments done more than a year later in December (after most of the work using mouse adipocyte suspensions)in bicarbonate buffers with lower glucose concentrations failed to produce rat adipocyte suspensions that were as responsive to insulin as had been previously found. It is possible that cell breakage was greater in this series of experiments (Table 13 B).

TABLE 13

The insulin effect in adipocytes from fed ♂ rats of mixed age, expressed as the percentage increase in the oxidation of glucose-1-^{14}C with insulin of the value without insulin.

A	Insulin Concentration U/ml.		
	$+10^{-4}$	$+5x10^{-4}$	$+10^{-3}$
+4% albumin	+30%(6)	-	+78%(6)
	-	+516%(3)	-
	-	+613%(3)	-
-4% albumin	-	+360%(2)	-
	-	+18%(3)	-

B	Insulin Concentration U/ml.		
$5x10^{-6}$	$5x10^{-5}$	$5x10^{-4}$	$5x10^{-3}$
-	+14 (3)	+13 (3)	-
-	+39 (4)	+31 (3)	-
+7 (3)	-	+29 (3)	-
-	+15 (3)	+1 (3)	+8 (3)
+12 (4)	+21 (4)	+19 (3)	-
+8 (5)	+20 (5)	+37 (5)	+33 (5)*

13A Adipocytes prepared from ♂ rats of mixed age with pure collagenase, were incubated at 37°C for 2-3 hrs. in bicarbonate buffer, pH 7.4 equilibrated with 95%O_2/5%CO_2, containing 0.2% glucose and 1-2μc glucose-1-^{14}C. The insulin effect is expressed as the increment in glucose-1-^{14}C oxidation calculated as a percentage of the basal value. The number of determination is given in parenthesis. The mean error in determinations of the response of adipocytes to insulin was ± 12%.

13B Adipocytes were prepared from 150-250 g. rats. The medium, containing 4% dialysed crystalline bovine albumin solution, had a glucose concentration of 0.1% except at * where it was 0.004%. Other experimental details as for Table 13A.

CHAPTER 10: The rate of production of $^{14}CO_2$ from glucose-I-^{14}C by adipocytes from fed ♂ lean (BSVS) mice of 5-6 months of age and cell lysis in adipocyte suspensions from lean and ob/ob mice.

This experiment showed that under the conditions adopted for incubation of adipocytes (see Ch.II, p.61-62), glucose-1-^{14}C oxidation was linear indicating that no lysis occurred during the incubation with hormone and glucose-1-^{14}C, (Fig. 13).

To determine whether cell lysis in adipocyte suspensions was a function of the preparative procedure, aliquots of adipocyte suspensions from lean and ob/ob mice were filtered over a silver mesh (pore size 20μ), washed twice with 10 ml. of isotonic albumin-free gassed bicarbonate buffer pH 7.4 and incubated with glucose-U-^{14}C by placing the mesh with its filtered adipocytes in the incubation medium. This has been shown to be a reliable method for separating intact from lysed cells (results not presented). Table 14 shows the percentage of glucose-U-^{14}C oxidised to $^{14}CO_2$ that could be attributed to filtered and therefore intact adipocytes in a few representative suspensions. It is clear that lysis of adipocytes was very variable. As it was unlikely to have occurred during the incubation with bicarbonate buffer containing 4% albumin and radioactive substrate, it must have been a result of the preparative procedure.

TABLE 14

The percentage of "intact" adipocytes in representative suspensions prior to incubations for metabolic studies.

		Glucose-U-^{14}C oxidation to $^{14}CO_2$	
Type of Animal	Age	No.of animals per suspension	Filtered / Unfiltered %
Lean	2-5 mths.	(20)	12%
		(20)	97%
ob/ob	2-4 mths.	(2)	33%
		(2)	35%
Lean	7 mths.	(17)	63%
		(20)	112%

Details are given in the text. Mice were of mixed sex.

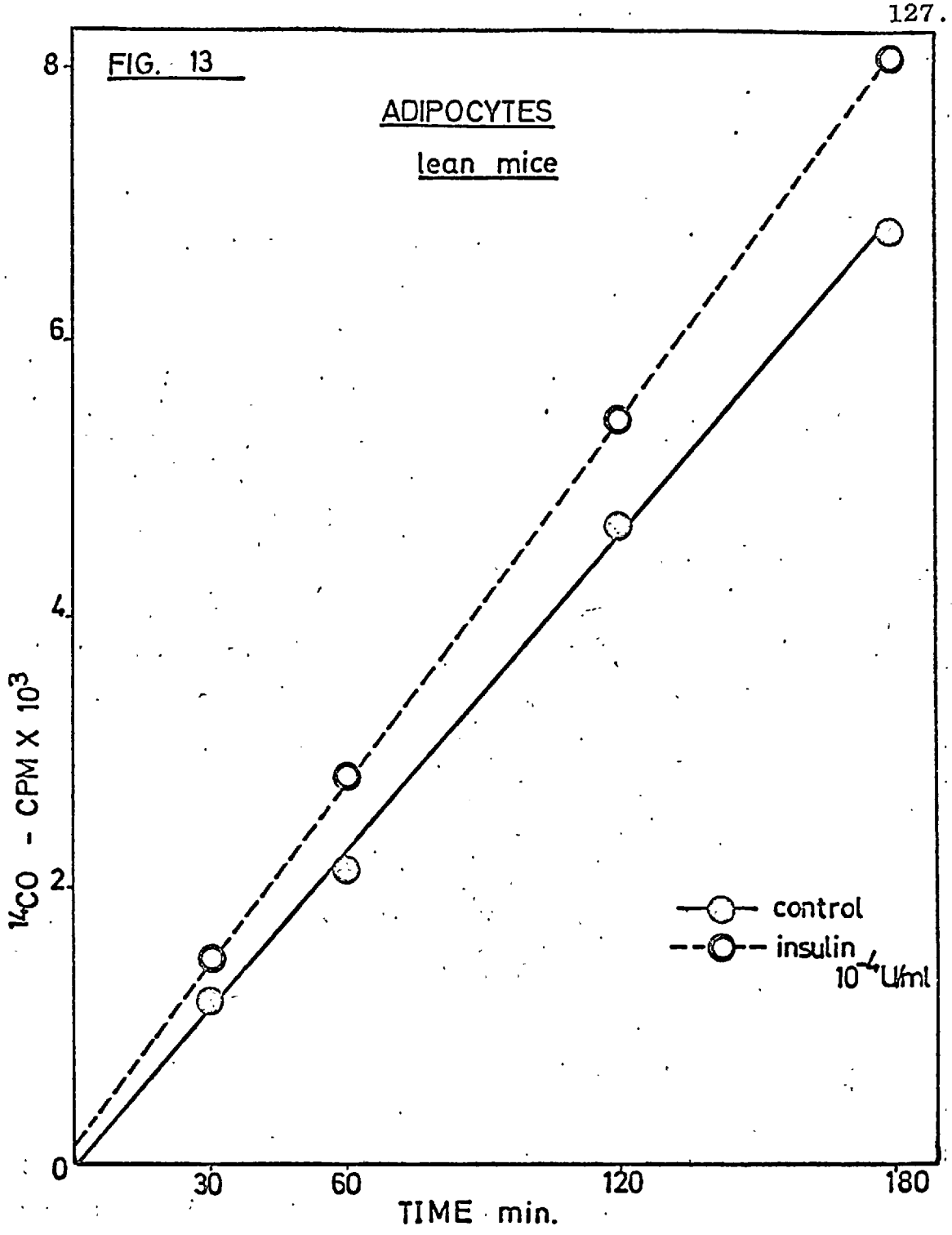


Fig.13 : The rate of oxidation of glucose-1-^{14}C in adipocytes from 3 month old ♂ lean mice and the effect of 10^{-4}U/ml. of insulin on this oxidation.

CHAPTER 11 : The response and sensitivity of isolated adipocytes prepared from the epididymal fat pads from 2-4 month and 6-8 month old lean, obese (ob/ob)mice and obese mice on a restricted diet (ob/ob-RD).

The insulin effect in adipoctyes prepared from epididymal fat pads from lean, ob/ob and ob/ob-RD mice of 2-4 months and 6-8 months of age at two glucose concentrations - (the sensitivity of adipocytes to insulin is dependent on the extracellular glucose concentration[92,93]) - and varying insulin concentrations is given in Fig. 14 (a) (b) and Fig. 15 (a) (b). The results were expressed as the percentage increase of glucose-1-^{14}C oxidised to $^{14}CO_2$ with different insulin concentrations over the value obtained with the same cell suspension incubated in the absence of insulin $\pm$ the standard error of the difference between the means for the control vial and that for the insulin vial. These were found to be 9.6%and 13.3% in lean and ob/ob adipocyte suspensions respectively. Gliemann[92] recorded a spread of $\pm$ 10% in the insulin values in the optimal range of the assay using four replicates. The variations in the responses to insulin in different adipocyte suspensions on different days were almost certainly due to variable cell lysis. It has been shown [172] that artificial lysis of fat cells resulted in a loss of response to insulin in proportion to the amount of cells broken. The rate of oxidation of the glucose-1-^{14}C and the effect of insulin on this oxidation was linear for all the adipocyte suspensions included in these results, indicating that no significant lysis was occurring during the incubation. Artificial lysis of the cells by ultrasonification resulted in

no significant glucose-1-^{14}C oxidation. The results are presented in graphical form in Figs.14(a)(b) and Figs.15(a)(b).

In Fig.14(a) it can be seen that with 0.2% glucose in the medium there was a statistically significant insulin effect in the adipocytes prepared from the fat pads of lean mice of 2-4 months of age at an insulin concentration of 5×10^{-5}U/ml. A maximum response was observed at 5×10^{-4}U/ml. in both the suspensions tested. The ob/ob mice in this group of experiments appeared to have a similar insulin effect, but the insulin sensitivity was slightly reduced so that a minimum insulin concentration of 5×10^{-4}U/ml. was required to give an effect. The maximum response obtained with adipocytes from 2-4 month old ob/ob mice was 5×10^{-3}U/ml.

In the presence of low glucose concentrations (0.03%) however, as shown in Fig.14(b), in two out of three cell suspensions prepared from lean mice at 2-4 months of age, the insulin effect was somewhat greater than that obtained with 0.2% glucose in the medium. Some insulin effect was observed with very low insulin concentrations (5×10^{-7} or 5×10^{-6}U/ml.) suggesting that these adipocytes may be more insulin sensitive at lower glucose concentrations[92,93]. A maximum response was obtained at a much lower insulin concentration viz. only 5×10^{-5}U/ml. The adipocytes from the ob/ob mice in this age group incubated with a low glucose concentration showed a smaller insulin effect and a minimum insulin concentration of 5×10^{-5} U/ml. was required to show a discernible effect which was not increased by higher concentrations of insulin. Thus the adipocytes from ob/ob mice in this age group, although more sensitive to insulin, seem to be slightly less responsive to insulin at a low glucose concentration than those from lean mice.

The results obtained with older mice at 6-8 months

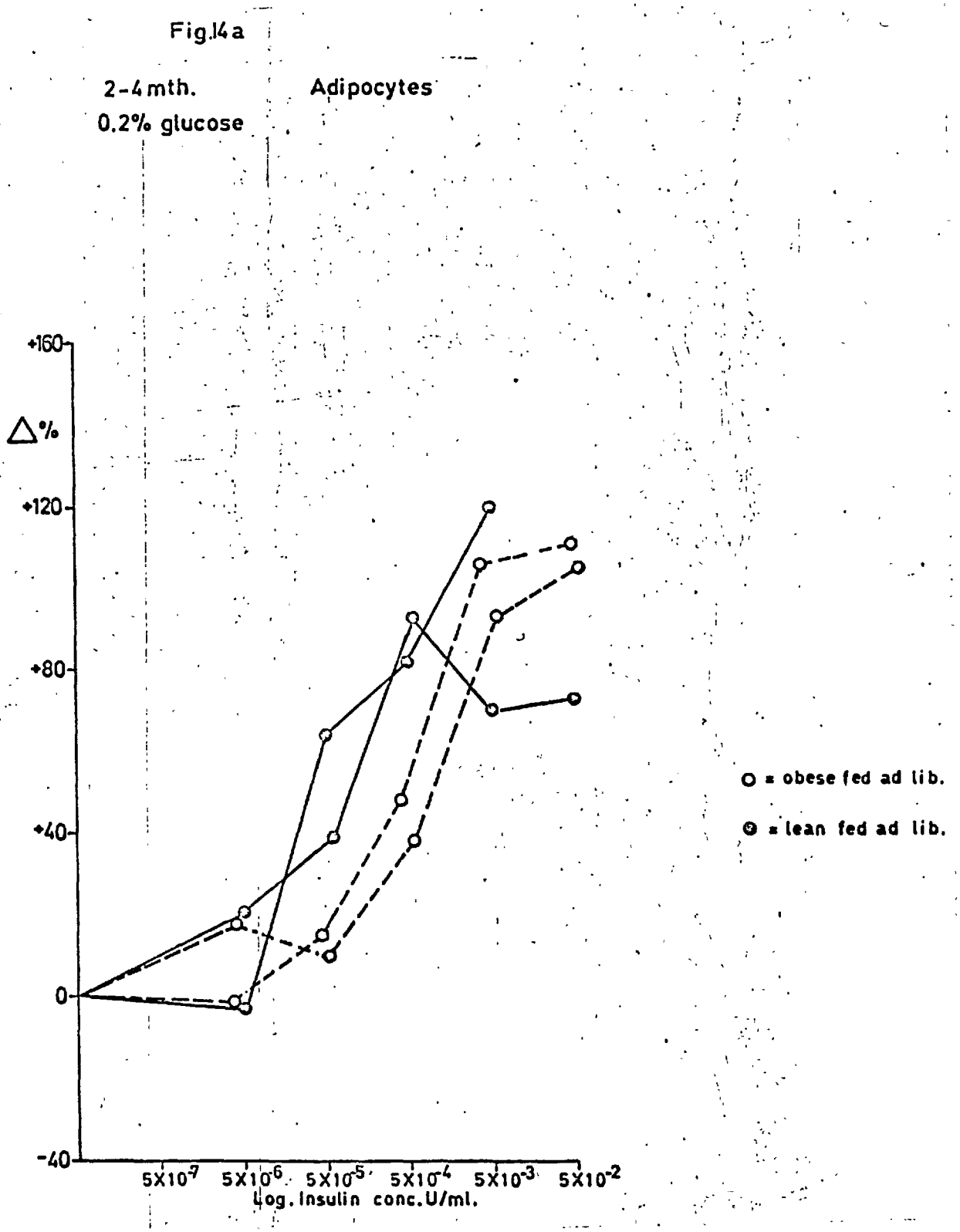


Fig. 14(a): Legend on p. 132

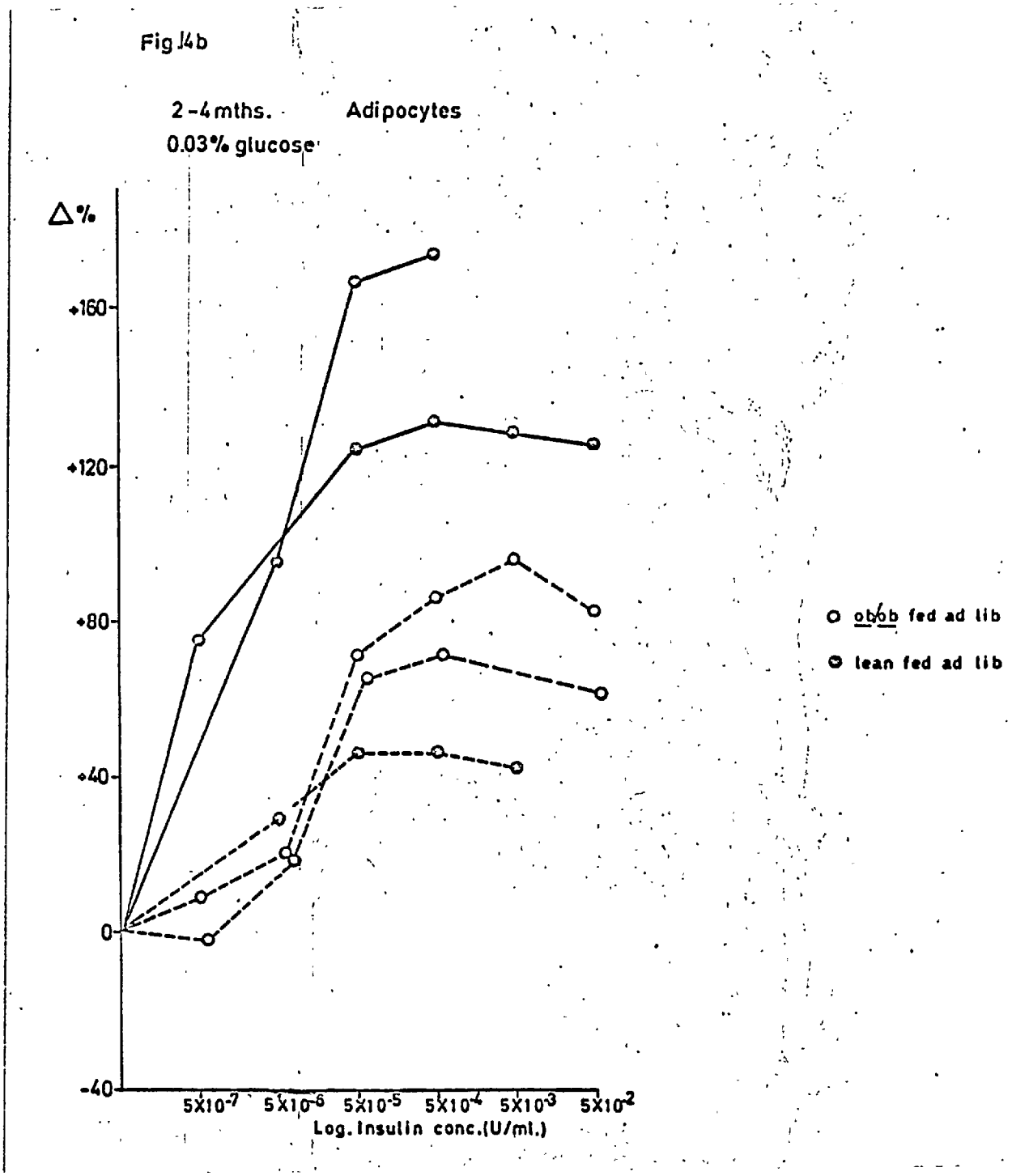


Fig. 14(b): Legend on p.132.

LEGEND TO FIG. 14(a)(b)

The insulin dose-response curves for suspensions of adipocytes prepared from 2-4 month old fed lean and obese (ob/ob) mice.

Adipocytes incubated in bicarbonate buffer, pH7.4 containing 1.2mM/L of Ca^{++}, 4% dialysed crystalline bovine albumin, 0.2% or 0.03% glucose and 1-2 µc glucose-1-^{14}C (sp. activity 1.60 µc/mg.), for 1 or 2 hours at 37°C in an atmosphere of 95%O_2/5%CO_2.

Results expressed as the increment in the oxidation of glucose -1-^{14}C to $^{14}CO_2$ in the presence of insulin as a percentage of the control value = $\triangle$%. About 15-20 lean and 1-3 ob/ob or ob/ob-RD mice were used for the preparation of each adipocyte suspension. Each curve given in the figure was obtained from a single cell suspension, and each point on the curve was obtained with 3-4 determinations, and the average error in these determinations (S.E.D.M.) was about ± 12%.

LEGEND TO FIG. 15(a)(b)

The insulin dose-response curves for suspensions of adipocytes prepared from 6-8 month old fed lean and obese (ob/ob) mice and from fed obese mice on a restricted diet (ob/ob-RD).

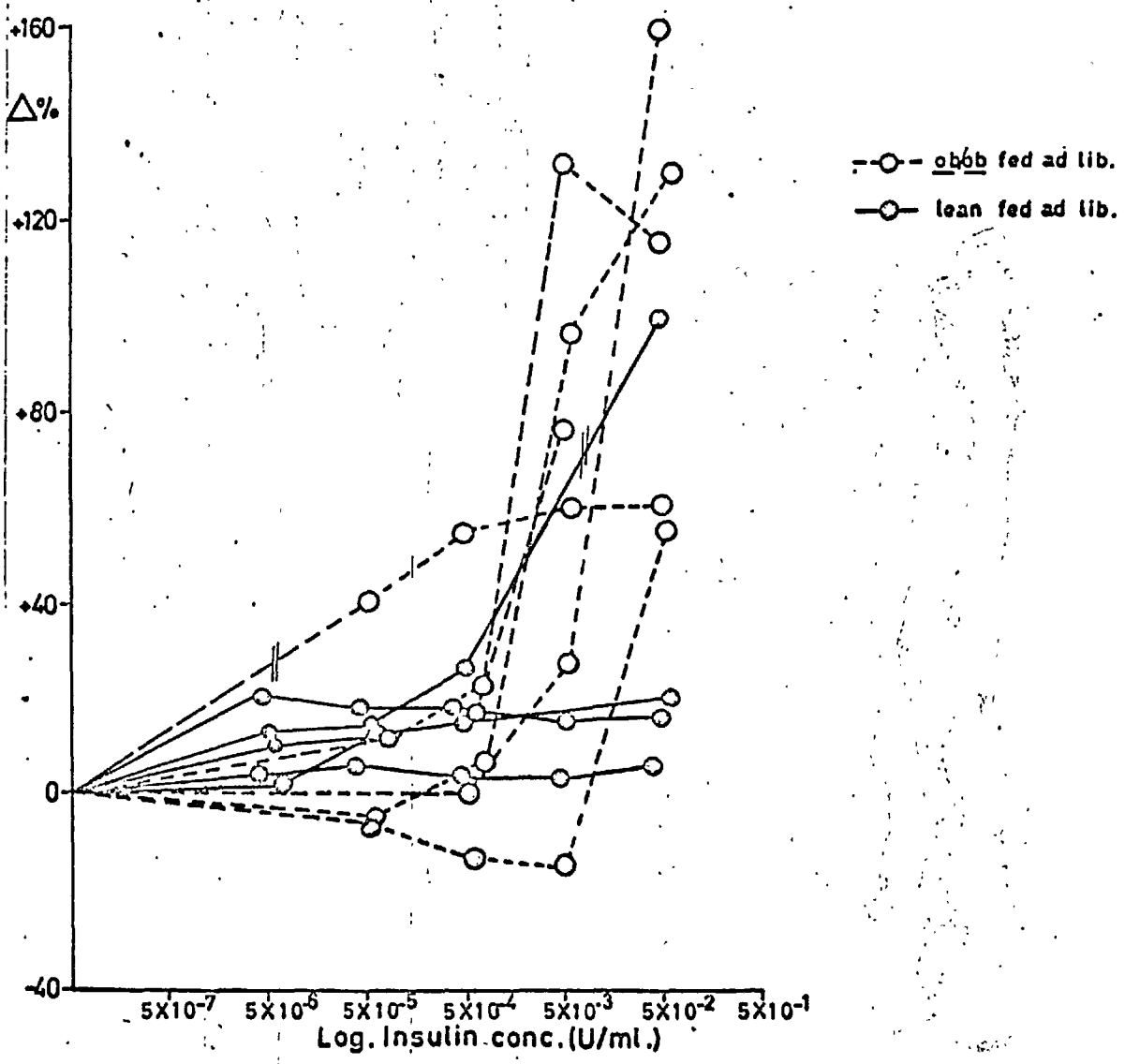
Incubation conditions and results expressed as in Fig. 14(a)(b).

About 15-20 lean and 1-3 ob/ob or ob/ob-RD mice were used for the preparation of each adipocyte suspension.

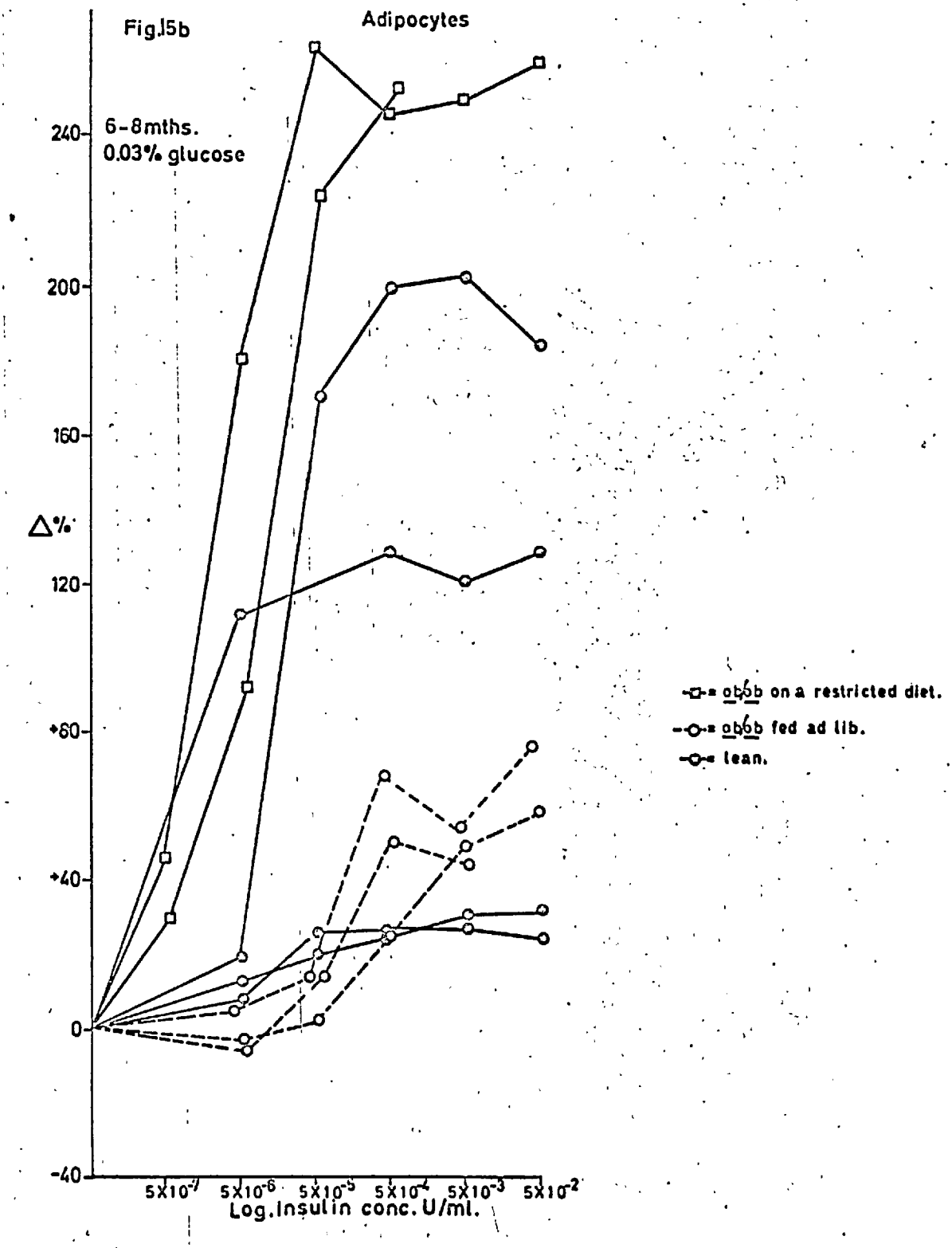
Fig.15a

6-8 mths. Adipocytes

0.2% glucose



Legend on p.132.



Legend on p.132

TABLE 15 : The response to insulin of suspensions of adipocytes prepared from ♂ 6-8 month old VMN-lesioned lean mice that became obese.

	Insulin Concentration U/ml.					
	5×10^{-7}	5×10^{-6}	5×10^{-5}	5×10^{-4}	5×10^{-3}	5×10^{-2}
Suspension 1	$+38 \pm 5(4)$*	$+71 \pm 8(4)$*	$+86 \pm 11(4)$*	$+79 \pm 6(3)$*	$+77 \pm 9(3)$*	-
Suspension 2	$+79 \pm 40(4)$	$+149 \pm 33(4)$*	$+167 \pm 59(3)$*	$+167 \pm 30(4)$*	$+163 \pm 31(3)$*	$+131 \pm 40(3)$*

* significantly different from control value $p < 0.05$

Incubation conditions and results expressed as in Fig. 14 (a) (b).

Two mice were used for each of the two suspensions, which were incubated in bicarbonate buffer containing 0.03% glucose.

of age are given in Fig. 15 (a) (b) and showed some differences from the 2-4 months age group. Thus, at the higher glucose concentration (0.2%) as shown in Fig. 15 (a) there was very little statistically significant response to insulin in any of the experiments with lean mice (only one out of this group showed a statistically significant response with 5×10^{-2}U/ml. of insulin). A reduction in the sensitivity and response to insulin with increasing age in adipocytes from rats has been recorded by Gliemann[92] and Gries[198]. In contrast, the cells from ob/ob mice responded to insulin in 5 out of 6 experiments at a concentration of 5×10^{-3}U/ml. and in all experiments at 5×10^{-2}U/ml. The maximum responses in this group were very variable and ranged from 55 - 160%. It should be noted that 5×10^{-2}U/ml. was the highest concentration of insulin tested and so it is conceivable that "maximum" responses were not in fact measured.

In experiments with a low glucose concentration (0.03%), shown in Fig. 15 (b), the results obtained with cells from lean mice were more variable. There was some insulin effect at 5×10^{-5}U/ml. in each experiment, whereas the cells from ob/ob mice were insensitive to insulin except in the presence of 5×10^{-4}U/ml. of insulin. Cells from the ob/ob-RD mice in this age group and in the presence of a low glucose concentration were extremely insulin sensitive, showing a significant effect with only 5×10^{-7}U/ml., and a maximum, extremely pronounced response was obtained with only 5×10^{-5}U/ml.

Adipocytes from 2-4 month old lean mice incubated with a medium glucose concentration of 0.1% showed a response and sensitivity to insulin that were between those seen with a medium glucose concentration of 0.03% and 0.2%. Table 15

shows the dose response values for the two adipocyte suspensions obtained from the four lean mice, lesioned in the ventromedial nucleus region of the hypothalamus, which became hyperphagic and obese. Both suspensions were markedly responsive to insulin, a significant effect occurring with as little as 5×10^{-7} or 5×10^{-6}U/ml. Nearly maximal effects occurred at between 5×10^{-6}U/ml. and 5×10^{-5}U/ml. of insulin. Thus, in terms of sensitivity, magnitude of response and concentration of insulin at which the maximal response occurred, adipocytes from hypothalamic lesioned obese mice were markedly different from adipocytes from ob/ob mice.

D. IN VIVO LEVELS OF BLOOD GLUCOSE AND PLASMA IRI

These experiments were initiated in order to test the hypothesis that the ob/ob mice, which are known to be hyperphagic, may show a number of abnormal parameters of intermediary metabolism simply because they are more frequently studied in a fully fed state, whereas the lean mouse, which has a feeding pattern with a definite circadian rhythm[196], may be more frequently studied in the postabsorptive state. Further, these experiments would be able to inform us of the comparability of the *in vitro* data on ob/ob and lean mice with the ob/ob-RD data which was obtained in the afternoon and where the animals were assumed to be in the postabsorptive state. Some of the animals used in this work were also used for the *in vitro* studies.

A decreased capacity to mobilise free fatty acid in epididymal adipose tissue from fasted ob/ob animals has been observed in both fat pads[57,58,199-201] and in isolated fat cells[202,203] and this mobilisation is relatively unaffected by adrenaline. This defect in lipolysis has been suggested as a primary cause of the obesity[31]. In a related study, plasma FFA concentrations were measured in order to ascertain whether these hyperinsulinaemic ob/ob mice mobilised FFA from adipose tissue in response to adrenaline and glucose. The results of this study, which are to be published, have not been presented here.

The experiments presented here were designed to investigate the effects on plasma IRI, blood glucose and liver glycogen of a controlled glucose load on the fasted state, the effects of fasting from a controlled fed state and the effects of various glucose loads on the fed state. The experimental design was chosen so as to show that differences between ob/ob and lean mice are larger in the fed ad libitum state and that small differences in the rates of removal or mobilisation of glucose (or free fatty acid) might be magnified by increased feeding in the ob/ob mice and may account for some of the abnormalities of the syndrome.

CHAPTER 12: Concentrations of plasma IRI and blood glucose in fed and fasted lean, ob/ob and ob/ob-RD mice.

In these experiments sampling of tissues did not occur at any particular time of the day but samples from all three types of mice were included in any one experiment to randomize this possible error[204].

Table 16 shows that obese mice in the fed ad libitum state had higher concentrations of blood glucose and plasma immunoreactive insulin in both age groups studied. The very high plasma concentration of IRI in older fed ob/ob mice confirms the levels reported by Westman[33a] with the same double antibody method[182,183] used here. Lower plasma IRI concentrations in ob/ob mice have been obtained[46,48] with a different double antibody immunoassay procedure[205] involving centrifugation

TABLE 16

Concentrations of Plasma Immunoreactive Insulin and Blood Glucose in Fed and 24 hour Fasted Mice

Blood samples were obtained from mice of mixed sex. Mice which were 6 hour fasted received 0.5 ml. 1M glucose intra-gastrically, to achieve an initial controlled "fed" state. Results are expressed as μU/ml. plasma (IRI; units of mouse pancreatic insulin) or mg/100 ml. blood (glucose). Mean values are given ± S.E.M. Number of observations in parentheses were equal to the number of mice used.

Mice	Age	Duration of Starvation (hours)	Plasma IRI	Blood Glucose
Lean	2-4	0	35 ± 10 (9)	148 ± 7 (10)
Lean	6-9	0	235 ± 45 (24)	109 ± 9 (16) *
ob/ob	2-4	0	1160 ± 460 (11) *	377 ± 58 (9) *
ob/ob	6-9	0	9450 ± 2050 (12)†**	313 ± 35 (15) **
ob/ob-RD	6-9	0	2140 ± 1080 (5)****	108 ± 4 (13)‡
Lean	2-4	6	< 35 (3)	147 ± 11 (6) ∫
ob/ob	2-4	6	235 ± 60 (4)∫	240 ± 24 (6) †∫ ∅
ob/ob-RD	2-4	6	< 35 (3)	217 ± 4 (3) ∅
Lean	2-4	24	< 35 (5)	81 ± 9 (5) *
Lean	6-9	24	< 35 (7)	58 ± 7 (11)**
ob/ob	2-4	24	70 ± 15 (6)	81 ± 11 (6) †
ob/ob	6-9	24	280 ± 100 (6) ∿	45 ± 6 (6)
ob/ob-RD	2-4	24	< 35 (3)	45 ± 6 (6)

* p≤0.05 Significantly different from fed lean 2-4 mth old value
** p≤0.001 " " " " " 6-9 " " "
† p≤0.05 " " " " ob/ob 2-4 " " "
‡ p≤0.05 " " " " " " 6-9 " " "
∿ p≤0.05 " " 24 hr fasted ob/ob 2-4 " " "
∫ p≤0.01 " " " " " value
∅ p≤0.01 " " from lean 6 hr. fasted value

rather than filtering of the γ-globulin-insulin complex. Plasma IRI also increased with age in lean mice. Blood sugar concentrations were lower in older lean mice.

Table 16 shows too that the high levels of blood glucose in ob/ob mice of both age groups were not evident in the 24 hour fasted state. The plasma IRI concentrations, however, although greatly reduced, were still significantly higher (particularly in the 6-8 month age group) than that of the lean mice after a 24 hour fast; this hyperinsulinaemia after 24 hour fasting of ob/ob mice was not seen by Westman[33a] or Stauffacher[48].

Evidence of the increase of the hyperinsulinaemia with age in ob/ob mice (reaching a peak at about 6-7 months of age), concomitant with the accretion of a pancreatic histology suggestive of insulin over-production[31,33a], has encouraged some investigators to suggest that hyperinsulinism is a secondary adaptation to the glucose intolerance of these mice. In an attempt to assess the significance of this hyperinsulinaemia, ob/ob-RD mice aged 6-8 months were investigated. Ob/ob-RD mice fed ad libitum were found to have significantly higher plasma IRI concentrations but normal blood sugar concentrations. If blood was taken when these mice were actually feeding (as opposed to the post-absorptive state during which sampling normally occurred), the plasma IRI concentration was about 4000 μU/ml. which was considerably higher than in lean mice which had received excess glucose (Table 17). The hyperinsulinaemia found concomitant with normal blood sugar concentrations was an interesting observation as these mice have been shown to be sensitive to the hypoglycaemic action of exogenously administered insulin[54]. In the younger age group, where plasma IRI was not so high, there

was no difference in plasma IRI between lean and ob/ob-RD mice after 24 hours fasting. Thus long-term partial food deprivation did not completely reverse the tendency to high levels of plasma IRI in ob/ob mice.

The concentrations of blood glucose and plasma IRI 6 hours after a small intragastric glucose load (administered so as to achieve an initial controlled "fed" state) are also shown in Table 16. This glucose load was not sufficient to significantly change the blood glucose of lean mice when sampled 90 minutes later and this therefore justified its use in ensuring that all these mice were in a "controlled fed" state (Table 17). After 6 hours fasting, the plasma IRI in young ob/ob mice had fallen from their normal fed ad libitum value of 1160 μU/ml. to 235 μU/ml. which was still significantly higher than 24 hour fasted ob/ob values and the values of both lean and ob/ob-RD mice, which reached fully fasted (24 hour) values after only 6 hours fasting. Rapid decline in immunoreactive insulin during starvation in hereditary obese mice has also been reported by Stauffacher[48]. Blood glucose levels of ob/ob mice were still elevated after 6 hours fasting. This was probably due to the same higher level of blood glucose these animals had when given the glucose load in the fed state (Table 17). 6 hours fasting was also not sufficient to reduce the blood sugar concentrations of lean and ob/ob-RD mice (2-4 months old) to 24 hour fasted levels.

CHAPTER 13: Concentrations of blood glucose and plasma immunoreactive insulin after intragastric glucose loading in fed ad libitum mice.

Table 17 shows the insulin and blood glucose responses to oral glucose in fed mice. Samples were collected between 60-90 minutes after the last glucose load in order to obtain conditions that were approaching a steady state. Even when the blood sugar of lean mice was thus raised so as to be comparable to that normally seen in obese mice (Table 16), the immunoreactive plasma insulin never exceeded 1000 μU/ml. in individual lean mice, whereas it exceeded 16000 μU/ml. in one group of obese mice. In obese mice there was no direct correlation between blood glucose and insulin level; the highest IRI level was seen after 3 doses of glucose spread over $4\frac{1}{2}$ hours although the blood glucose was no higher than in experiments of shorter duration. Similar findings have been reported by Genuth[46] who loaded the mice with intraperitoneal injections of glucose.

The remarkable ability of the liver of the ob/ob mouse to show a net synthesis of glycogen, closely related to the concurrent blood glucose concentration, is also shown in Table 17 (data of Miss Jennifer Elliott).

CHAPTER 14: Concentrations of plasma IRI and blood glucose after intragastric glucose loading in 24 hour-fasted mice.

The measurement of the response of plasma IRI and blood sugar to a standard intragastric glucose load in 24 hour fasted mice served to indicate to what extent the hyperinsulinaemia of ob/ob mice was dependent on glucose as a stimulus and whether a more rapid rate of synthesis and secretion

TABLE 17

Concentrations of Plasma Immunoreactive Insulin and Blood Glucose and of Liver Glycogen in Fed Mice

Fed female mice aged 6-8 months received intragastic glucose. Blood was taken after 60-90 minutes. When three doses of glucose were given, these were at 90 minute intervals. Results are expressed as μU/ml. plasma (IRI; units of mouse pancreatic insulin), mg/100 ml. blood (glucose) and μmoles/g fresh liver (glycogen). Mean values are given $\pm$ S.E.M. Number of estimations in parentheses were equal to the number of mice used.

Mice	Dose of Glucose	Delay before Sampling (min.)	Plasma IRI	Blood Glucose	Liver Glycogen
Lean	1 ml 2M	60	270 $\pm$ 70 (3)A	329 $\pm$ 42 (4) G	-
ob/ob	1 ml 2M	60	>11,000 (3)B	940 $\pm$ 200 (3) H	-
Lean	1 ml 2M	75	-	185 (2) I	145 $\pm$ 35 (3) M
ob/ob	1 ml 2M	75	-	385 $\pm$ 25 (3) J	705 $\pm$ 60 (3) N
Lean	1 ml 2M (three times)	90	690 $\pm$ 200 (3)C	261 $\pm$ 9 (3) K	285 $\pm$ 30 (4) O
ob/ob	1 ml 2M (three times)	90	>16,000 (3)D	595 $\pm$ 30 (3)L	1420 $\pm$ 45 (3) P
Lean	½ ml M	80	35 (3)E	126 $\pm$ 3(3)Q	
ob/ob	½ ml M	80	5100 $\pm$ 4100(3)F	554 $\pm$ 130(3)R	

Significantly different at $p<0.001$ KQ, KL
$p<0.01$ JL, GH, GQ
$p<0.05$ MN, OP, EC, AE, QR, HJ

of insulin occurred. In an attempt to mimic the effects of feeding sufficient to re-establish the fed state after a fast, two 1 ml. loads of 2M glucose separated by a 90 minute interval were administered intragastrically to 24 hour fasted mice; control mice were intubated in the same manner with 1 ml. 0.9% saline. The plasma IRI concentrations of the fasted and "refed" mice are shown in Table 18.

It can be seen that the glucose loads did not have a significant effect on the plasma IRI of lean mice when this was sampled 3 hours after the start of the intubations. It is more than probable that any rise of plasma IRI occurring after the second glucose load was over by the time sampling started 90 minutes later. However, the two intragastric intubations of glucose appeared to result in extremely higher, though variable levels of plasma IRI in ob/ob mice, the control 24 hour fasted value of 95 rising ten-fold to 1030 μU/ml. detectable 90 minutes after the last glucose load.

In order to assess whether hyperinsulinaemia was secondary to massive obesity, the plasma IRI response to glucose was also measured in ob/ob-RD and very young ob/ob mice. The exaggerated response of the plasma IRI of 6-8 month old ob/ob mice was not seen in these two groups, the absence of a persistent hyperinsulinaemia being particularly evident in the ob/ob-RD mice 6-8 months old.

Finally, the importance of the oral route of glucose administration compared to the parenteral was investigated by giving 6-8 month old ob/ob mice two doses of subcutaneous glucose equal to those given in the intragastric glucose experiments. There was a small rise in plasma IRI in

TABLE 18

Influence of Glucose on Plasma Level of Immunoreactive Insulin in Fasted Mice

Mice of mixed sex were fasted for 24 hours. Then 1 ml. 1M glucose, or 0.9% sodium chloride was twice administered intra-gastrically, with a 90 minute interval. After a further 90 minutes, plasma level of IRI was determined. In one experiment, glucose was administered subcutaneously (doses as above). Results are expressed as μU/ml. plasma (IRI; units of mouse pancreatic insulin). Mean values are given, $\pm$ S.E.M. Number of observations in parentheses. Number of mice used in circles.

Mice	Age (mths.)	Treatment	Plasma IRI	
Lean	6-8	Saline	<35 (5)	⑫
Lean	6-8	Glucose	45 ± 30 (5)	⑨
ob/ob	6-8	Saline	95 ± 80 (4)	⑨
ob/ob	6-8	Glucose	1030 ± 570 (6)	⑥
ob/ob-RD	6-8	Glucose	78 ± 30 (6)	⑥
ob/ob	6-8	Glucose (Subcutaneous)	380 ± 90 (3) *	③
ob/ob	1-2	Glucose	165 ± 90 (3)	③

*Significantly different from lean 6-8 month glucose value $p \leq 0.01$

this experiment which, although eightfold greater than the values observed in lean mice, was still smaller than that observed in ob/ob mice fed intragastrically.

Blood glucose concentrations were variable in this experiments (110-160 mg./100 ml. in both groups) but in parallel experiments done on 2-4 month old female mice (results not shown) the blood sugar 90 minutes after the second glucose load was 152 and 163 mg.% in lean and ob/ob mice respectively, compared to 24 hour fasted, saline-intubated values of 91 and 91 mg.% respectively; (however, a higher rate of glucose absorption from the alimentary tract has been suggested to occur in ob/ob mice[206]). This indicates that the hyperinsulinaemia of 6-8 month old ob/ob mice was persistent 90 minutes after the last glucose load when blood glucose concentrations were not markedly elevated.

E. BODY COMPOSITION

CHAPTER 15: To determine the degree of obesity in lean and ob/ob mice and in goldthioglucose-induced obesity.

Obesity has been defined as the ratio of the fat:fat-free dry weight of an animal[207]. Table 19 shows the extractable fat and water contents of the carcass as a percentage of the body weight and also the nitrogen in the fat-free dry residue of the carcass. It shows clearly that the ob/ob mice have a greatly increased percentage of body fat[34,130] and this can almost entirely account for their excess body weight. Mice made obese by intraperitoneal injections of goldthioglucose had a similarly raised fat content in the carcass[145] but this was significantly less than that found in ob/ob mice at the same age(Table 19), as was also their final body weight (Table 1).

As a percentage of the body weight, the water content of course fell in both ob/ob[131] and goldthioglucose-obese mice, (again the fall was greater in the ob/ob mice). However, the water content of the carcass of ob/ob mice did increase by about 15% (as did the plasma volume (Table 20)), although this increase was not statistically significant. If one assumes that this compensatory rise also occurred in the lean body mass which is largely water, then it is evident that much of the weight of the excess lipid stored must be "metabolically inert"[209]. The rise in the water content of carcasses of goldthioglucose mice was also not significantly different from the lean value.

The nitrogen contents of the fat-free dry residue from lean, ob/ob and goldthioglucose mice were not significantly different from each other.

TABLE 19 : Body composition data in carcasses from lean, ob/ob and goldthioglucose obese mice.

	Fat as % body wt.	Fat:fat-free dry weight	H_2O gm.	H_2O as % body wt.	N as % of fat-free dry wt.
Lean	9.64 ± 0.62(11)	0.50 ± 0.04(5)	20.8 ± 0.5(7)	69.1 ± 0.61(7)	10.45(2)
ob/ob	48.4 ± 1.5(10)	5.1 ± 0.8(10)	23.8 ± 1.9(7)	34.1 ± 1.9(7)	10.45 ± 0.18(10)
Goldthioglucose-obese	28.9 ± 2.0(8)	1.53 ± 0.2(8)	22.3 ± 0.8(9)	50.8 ± 0.8(9)	10.9 ± 0.3(9)

The comparisons in columns 3 & 5 were not significantly different from each other.
All other comparisons significantly different from each other. $p < 0.01$.

F. CELLULAR CHARACTERISTICS OF INTACT EPIDIDYMAL FAT PADS

In this chapter measurements on the water and nitrogen content, the ^{3}H-Sorbitol space *in vitro*, the ^{125}I-albumin space *in vivo* of the epididymal fat pad, the number and size of its constituent adipocytes and the plasma volume were made in lean and *ob/ob* mice of mixed age.

The very reduced metabolism *in vitro* seen in Ch.B8 (Fig. 10 (a) & (b)) and also reported by other workers [55-58] could also be explained by a different structure and/or composition in this tissue. Other authors have commented on the abnormal histology of the adipose tissue of obese-hyperglycaemic mice[132,31]

CHAPTER 16: Cellular characteristics of intact epididymal fat pads relating to composition.

It is appropriate at this point to mention the marked macroscopic differences in appearance that pads from *ob/ob* mice displayed. Whereas fat pads from lean mice were usually either thin and white, usually relatively bloodless and well-filled with fat, fat pads from *ob/ob* mice were often yellowish in colour (especially in older mice) and gelatinous in texture with an increased fragility resulting in early loss of the form and shape of the pad on handling. Fat pads from lean mice hardly ever weighed more than 0.5 g., whereas pads from *ob/ob* mice weighed between 1 - 5 g. depending upon the age of the animal; (each pad from 6-8 month old *ob/ob* mice weighed an average of 4.02 ± 0.45g.(12)).The fat pad of the *ob/ob* mouse was further characterised by small brownish-yellow inclusions,

(0.3 cm. - 0.8 cm. in diameter), sparsely distributed (1-4 per pad) near the base of the fat pad. These were never investigated but have been commented on by Hausberger[132]. Their gelatinous, fatty nature makes it seem possible that they are either brown adipose tissue or an accumulation of the necrotic, fatty non-cellular cysts that are known to exist in the fat pads of these mice[132]. They were excluded from both the fat pad and adipocyte preparations on all occasions. The fat pads from ob/ob-RD mice were similar qualitatively but were slightly reduced in size. The most striking feature of these pads was the extraordinary leaking of their contained fat. This was visible as an oily 'sweat' glistening on the cut surface of the pad and was left behind on the torsion balance and on the forceps used to transfer the pieces to the incubation vials. The change in the torsion balance tare could be up to 30% of the weight of a pad after a single weighing and this certainly was a significant contribution to the error in the values for glucose-1-^{14}C oxidation in pads from this group of mice.

In Table 20 are shown some of the cellular characteristics of the epididymal fat pads from lean and obese mice. No significant difference was observed in the nitrogen content per unit wet weight of pad between pads from lean and obese mice either at 2-4 months or at 6-8 months of age, in agreement with some previous studies[31,56,210], although Marshall et al.[58] have reported it to be 30% of the lean value. The nitrogen values in Table 20 also show a significant rise in the nitrogen content of the obese fat pad with age; Christophe[56] found that the percentage of protein in the pads of heavier animals may even exceed values found in lean mice. The nitrogen

TABLE 20

Cellular Characteristics of Lean and Obese Epididymal Fat Pads

	2-4 months		6-8 months		
	Lean	ob/ob	Lean	ob/ob	ob/ob-RD
Water content µl./mg.	-	-	0.081 ± .008 (6) A	0.037 ± .004 (22) ⑦ B	-
^{125}I-Albumin space µl./mg.	-	-	0.029 ± .002 (13) ⑦ C	0.007 (4) ② D	-
^{3}H-Sorbitol space µl./mg.	-	-	0.042 ± 0.008 (5) ⑥ E	0.046 ± 0.0003 (8) ③ F	-
Nitrogen content γ N/mg.					
< 300 mg. pad	-	2.09 ± 0.09 (6) G	2.78 ± 0.17 (29) H	2.42 ± 0.24 (12) I	1.87 ± 0.20 (4) J
> 300 mg. pad	1.77 ± 0.14 (4) K	1.52 ± 0.06 (15) L	2.03 ± 0.16 (8) M	2.30 ± 0.22 (12) N	1.36 ± 0.11 (15) O
No. of cells per mg.	2270 ± 285 (8) ④ P	605 ± 50 (12) ⑥ Q	-	-	-
Plasma volume ml. ≠	-	-	1.49 ± 0.05 (12) R	1.99 ± 0.16 (5) S	-

Difference significant $p \ll 0.001$ AB, GL, NL, PQ. $p \ll 0.05$ HM, JO. $p \ll 0.01$ MO, NO, RS.

≠ 9 - 12 months old animals, fed and fasted pooled.

Legend on p. 153.

LEGEND TO TABLE 20

Experimental details are given in the text.
Values are given as the means ± the S.E.M. with the number of determinations in parenthesis. These were equal to the number of animals used, except where these have been given in circles. The nitrogen content was determined on pads of differing wet weight and the results presented in two groups based on the wet weight of the pads.

content of pads (>300 mg.) from ob/ob-RD mice was found to be significantly reduced and was less than that found in lean or obese mice at 6-8 months of age. In young mice the number of adipocytes per unit wet weight of fat pads from lean mice was nearly fourfold greater than in obese mice. The plasma volume of obese mice was found to be slightly but significantly higher than that found in lean mice.

It can also be seen that the water content and *in vivo* ^{125}I-Albumin space per mg. wet weight of epididymal fat pad were also reduced proportionally in obese mice of 6-8 months of age. However, no significant difference in the ^{3}H-Sorbitol space between lean and obese mice was detected when pads were incubated in bicarbonate buffer for 60 minutes *in vitro*.

CHAPTER 17: The size of adipocytes in intact epididymal fat pads and in cell suspensions prepared from epididymal fat pads in lean and ob/ob mice.

As the triglyceride content of an adipocyte varies with age[211] and as one could expect ob/ob adipose tissue to have much more triglyceride per cell [31,277], an attempt was made to use nitrogen or DNA as an index of cell number. To this end, albumin was omitted from the incubation with purified collagenase in the preparation of adipocytes. However, the variable fragility of mouse cell suspensions, in particular those of ob/ob mice (see Table 14) made nitrogen or DNA inadequate as parameters of intact cell number. The absence of albumin could well have contributed to the instability of mouse fat cells during their preparation[93]; once in 4% albumin, linear rates for glucose-1-^{14}C

oxidation to $^{14}CO_2$ with time were obtained (Fig. 13), indicating that no lysis occurred during the incubation with insulin. However, Lech [171] has shown that the nitrogen in the suspending protein-free medium rarely exceeds a third of the protein of the entire cell digest when rat adipocytes are prepared with purified collagenase in albumin-free buffer. It is probable that mouse adipocytes are more unstable than rat adipocytes.

In order to obtain further evidence for this, adipocytes in photomicrographs of suspensions from pads of lean and ob/ob mice were sized with a particle size analyzer and compared with similar values from photomicrographs of fixed adipocytes prepared according to Hirsch[180a]. Fixed adipocytes from intact epididymal fat pads of ob/ob mice were very much larger than those from pads of lean mice (Fig. 16). However, when suspensions of adipocytes were prepared by collagenase digestion there was a reduction in the mean size of the population of particles counted and this was particularly striking with suspensions from ob/ob mice, indicating that the larger cells of these animals were more vulnerable to lysis by the preparative procedure.

As it was impossible to distinguish small adipocytes unequivocally when adipocytes prepared with collagenase digestion were fixed with OsO_4 for between 3 and 24 hours (as the characteristic deformation of the cell membrane of intact adipocytes did not occur) the particulate material smaller than 30μ most probably did not consist of intact adipocytes. The true sizes of ob/ob and lean adipocytes prepared by this method were probably masked by the large percentage of broken material at the lower end of the distribution profile which suggests that in the three suspensions used for these photomicrographs, considerable lysis had occurred during the preparation of the suspensions.

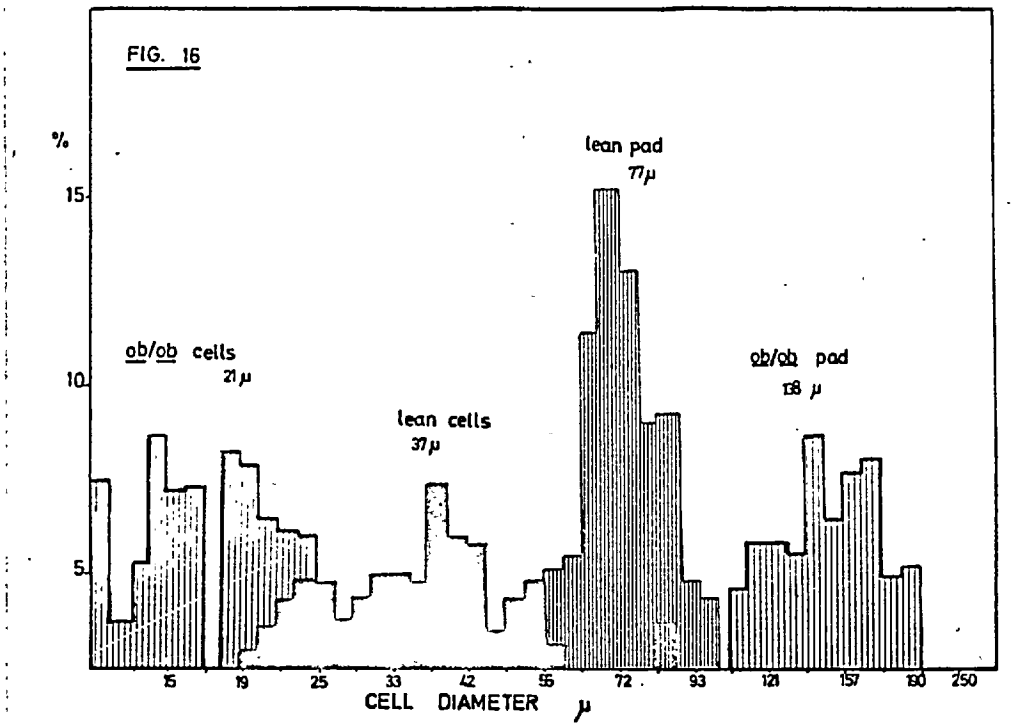


Fig. 16: Histogram of percentage distribution of diameter of fixed adipocytes from ♂ lean and ob/ob mice 2-4 months old.

Pad: Fixed cells prepared by incubation with 2%OsO_4 from intact pads by method of Hirsch[180a].

Cells: Prepared by digestion with collagenase, followed by fixation with 2%OsO_4 for between 3-24 hrs. 8 pads from lean mice and 12 from ob/ob mice were used to obtain the data for cell size in intact pads. 3 suspensions each from lean (45 mice) and ob/ob mice (9) were used for the data on adipocyte suspensions. 340 determinations were made on cells from pads from lean mice, 480 from pads from ob/ob mice, 1420 from cell suspensions from lean mice and 1820 from cell suspensions from ob/ob mice. The median size of the 4 populations is given in the figure.

Ordinate: The number of particles sized as a percentage of the total number sized after multiplying by a correction factor.

Abscissa: Particle diameter in μ on an exponential scale.

CHAPTER IV

DISCUSSION OF RESULTS

A. Diaphragm muscle *in vitro*.

B. Epididymal fat pads *in vitro*.

C. Isolated adipocytes *in vitro*.

D. *In vivo* levels of blood glucose and plasma IRI.

E. Cellular characteristics of intact epididymal fat pads.

A. Diaphragm Muscle in Vitro.

The results shown in Chapter III A1 & A2 confirmed that optimal conditions for incubation of mouse diaphragm *in vitro* were being used.

In Chapter III A3, the results obtained with the isolated mouse diaphragm and slices of mouse liver, using the technique of quantitative bidimensional autoradiography[107] and the computerized scanning method developed in this Department[165] were described. The qualitative pattern of glucose metabolism in the diaphragm and liver of the mouse were shown to be similar to those previously obtained with rats[107,212]. The quantitative values for glucose conversion into glycogen and the oligosaccharides (Method C), lactate and CO_2 were 2-5 times higher in mouse hemidiaphragm muscle than comparable values from rats obtained by an identical procedure[213]. This would be expected for tissues from a much smaller animal which,

on a weight basis, has a relatively higher BMR and food intake. The incorporation into $^{14}CO_2$ was higher and into lactate lower than that recorded by Hellman[63], using the same buffer and incubation techniques. No qualitative differences in the glucose incorporation into the above radioactive metabolites were observed in incubated liver slices or diaphragm muscle from lean or obese mice 6-8 months old. No significant difference in the incorporation of glucose-U-^{14}C into $^{14}CO_2$, alanine, lactic acid, glutamine and glutamic acid by lean and ob/ob diaphragms has been reported[63].

In agreement with the findings of Beloff-Chain[107, 213] the addition of 10^{-2}U/ml. of insulin affected only the soluble glycogen and oligosaccharide fraction of the aqueous ethanolic extract of diaphragm muscle: the insulin effect obtained in this report was however smaller than that obtained with rat diaphragm. No insulin effect was obtained with any metabolites extracted from incubated liver slices, as has been previously shown with rats[212].

Van Heijningen[214] has shown that insulin affects only the so-called "free" fraction of glycogen (viz. that which is extracted by trichloroacetic acid at room temperature); whereas the residual insoluble glycogen was uninfluenced by insulin. The incorporated radioactivity in the residue remaining after extraction with aqueous ethanol was shown to be glycogen as it was converted almost quantitatively into glucose after hydrolysis, and was also uninfluenced by 10^{-2}U/ml. of insulin (Table 6). However, when the increment was expressed as a percentage of the value in the absence of insulin, a clear response to 10^{-2}U/ml. insulin could be seen in hemi-

diaphragms from fed and fasted lean mice 6-8 months old (Table 7), in agreement with the original work of Beloff-Chain et al.[107].

Using mouse hemidiaphragms in phosphate buffer, a clear increment with insulin (expressed as a percentage of the basal value) in the glucose-U-^{14}C incorporation into total glycogen or "soluble" glycogen and oligosaccharides was seen in tissues from fed lean mice, 6-8 months old. No significant difference in the effect between diaphragm muscle from fed or 24 hour fasted 6-8 month old lean mice was seen, suggesting that the initial glycogen content of the diaphragm muscle does not affect the insulin effect on glucose-U-^{14}C incorporation into glycogen[197]. Although the difference was not significant it did appear that hemidiaphragms from 2-4 month old lean animals were slightly more responsive than hemidiaphragms from 6-8 month old lean mice. This latter tissue was also slightly more insulin responsive than the tissue from 6-8 month old obese mice. However, a definite effect of about +45% was obtained with hemidiaphragms from obese mice of this age with 10^{-2}U/ml; their diaphragm muscle was therefore definitely responsive to insulin.

In liver slices from fed lean 6-8 month old mice, 10^{-2}U/ml. of insulin did not affect the incorporation of glucose-U-^{14}C into soluble or insoluble glycogen, intracellular glucose, lactate or CO_2. Although much of the pathology of the ob/ob syndrome is thought to reside in the liver, further work with this tissue in this particular *in vitro* system was curtailed because of the fragility of liver slices from these mice, making the preparation unreliable and difficult to work with.

Information on three variables was obtained from the experiments from Chapter III A5-6; the basal level of incorporation of glucose-U-^{14}C into glycogen in diaphragm muscle in the

absence of insulin and the response and sensitivity of this incorporation to different concentrations of insulin in the incubation medium.

The results clearly showed that only in young, 2-4 month old ob/ob mice was the incorporation of glucose into glycogen significantly less than in lean mice of the same age. In fact, this reduction in glycogen synthesis was particularly significant as the dry weight of diaphragm from ob/ob mice is 25% more than those from lean mice[59] suggesting extracellular dehydration of this tissue as the intracellular potassium concentration is unchanged in these diaphragms[215]. No significant difference in the basal incorporation of glucose into glycogen in muscle from lean and ob/ob mice was noted either at 6-8 months of age or at about 12 months of age. Similar findings have been reported by Hellman[63] for glucose utilization and by Chlouverakis[55] for glucose uptake and glycogen synthesis.

Thus the incorporation of glucose-U-^{14}C into diaphragm glycogen increased with age in ob/ob mice, although it did not alter in lean mice. Gershoff[50], however, in in vivo experiments found that the incorporation of glucose into glycogen decreased with age in the ob/ob mice, whereas in agreement with the present studies it did not change in the lean mice. The increase in glucose-U-^{14}C incorporation into glycogen with age in ob/ob mice appears to be an adaptation to chronic hyperglycaemia as it was not seen in diaphragm muscle from 6-8 month old ob/ob-RD mice with normal blood sugar concentrations.

During the work on *in vivo* concentrations of plasma insulin and blood glucose, the opportunity to study basal glucose incorporations into glycogen was taken under controlled "fed" and 24 hour fasted conditions. One would have expected the initial glycogen content of the diaphragm to strongly influence the basal incorporation of glucose-U-^{14}C into glycogen as it does net glycogen synthesis, although the insulin effect on this incorporation was not affected by the initial glycogen content of the diaphragm[197] (Table 7). As was shown in Table 10 (p.102) feeding did probably lower the basal incorporation of glucose-U-^{14}C into glycogen, which strongly suggested that the reduced incorporation observed in quarterdiaphragms from fed 2-4 month old *ob/ob* mice reflected their increasing hyperphagia at this age, as they would have been studied more often in a "recently fed" state. After fasting *ob/ob* mice for 24 hours, the basal glucose-U-^{14}C incorporation into glycogen returned to a normal value (when the glycogen contents of *ob/ob* and lean diaphragms are similar[59]), following the slow disappearance of their hyperglycaemia during fasting (Table 16, p. 140).

Adaptation to their now stable hyperphagia at 6-8 months of age

was the probable reason for the normal values of glucose-U-^{14}C incorporation into glycogen seen in ob/ob mice at this age. Thus it is unlikely that the reduced glucose-U-^{14}C incorporation of muscle in ob/ob mice in the lipogenic phase is of significance as a primary cause of their hyperglycaemia, as it is more easily explained by their concurrent hyperphagia and glucose intolerance, and their relative inactivity (which would cause the lack of a "muscle activity factor" known to increase the glucose uptake of rat diaphragm *in vitro*[216]).

Most diaphragm bioassays have shown that the dose-response curve is linear between about 10^{-5} and 10^{-3}U/ml. of crystalline insulin[108,109,111,112,115]. Wardlaw[114] found the maximum insulin effect in hemidiaphragms from lean mice occurred at a concentration of 10^{-3}U/ml. A maximum effect of insulin was obtained in the present studies in this range, viz. 5×10^{-3}U/ml. in lean mice of both age groups tested.

Unlike the results reported on in *in vitro* experiments with the epididymal fat pad, no significant decrease in sensitivity or response to insulin was detected in the present experiment in quarterdiaphragms from lean mice with increasing age up to 8 months. Wardlaw[114] has reported that smaller mice possessed hemidiaphragms that were more responsive to insulin. The discrepancy between these results and those of Wardlaw can be explained by the fact that at the youngest age examined in this study, 2 months, lean mice weighed about 30 g., by which weight the age-dependent insulin response of mouse hemidiaphragms has stabilised[114]. The variability of response in hemidiaphragms from fasted lean mice, 10-20 g. in weight, reported by different investigators ranges from +60%[114] to +150%[217] with a medium

glucose concentration of about 0.1%.

Considerable difference, though, was seen in the maximal response to insulin (viz. at 10^{-2}U/ml.) in lean mice 6-8 months old (Fig. 9 (b)), and in mice 12 months old (Table 9); hemidiaphragms from the latter responded to insulin with an increment that was 180% of the basal incorporation in the absence of insulin. In view of the unchanged sensitivity and response to insulin with age in adult animals *in vitro* in the experiments mentioned above, the increased response seen in the experiments with older 12 month old mice could be ascribed to two changes in the experimental conditions: either to the higher Ca^{++} concentration of the incubation medium that was in use at that time or to the use of hemidiaphragms instead of quarterdiaphragms. The latter is an unlikely reason as it has been shown that hemidiaphragms are as responsive to insulin as quarterdiaphragms[114] (*v.s.* Chapter II p. 53).

In agreement with other findings in diaphragm[55] and abdominal muscle[55] of fasted mice at 3-5 months of age, the insulin effect, as measured either by an increase in the incorporation of glucose into glycogen or by an increment as a percentage of the basal value, was reduced in diaphragm muscle from *ob/ob* mice. This difference was more marked in the older animals so that only a very small insulin effect was apparent in muscle from 12 month old *ob/ob* mice. That the maximum responsiveness of diaphragm muscle from 6-8 month old *ob/ob* mice was severely limited was shown by the fact that increasing the insulin concentration in the incubation medium to as high as 5 x 10^{-2}U/ml. did not increase the insulin effect (Figs. 8(b) 9(b)). Quarterdiaphragms from *ob/ob* mice did, however, show

a greater responsiveness to 10^{-4}U/ml. of insulin (the minimum dose that resulted in a maximal response) at the younger age group tested. As the basal incorporation of glucose-U-^{14}C into diaphragm glycogen was reduced in ob/ob mice of this age group, the insulin effect, calculated as a percentage increase of the basal incorporation, was not significantly different from that in lean mice of the same age, although it appeared reduced. (Fig.9(a)).

Using an in vivo technique in which glucose-U-^{14}C was introduced by intraperitoneal injection[86], Stauffacher[59] showed almost complete unresponsiveness to as much as 10^{-2}U/ml. of insulin in the labelling of diaphragm muscle glycogen in 8 week old fasted ob/ob mice. However, 8 week old fasted Swiss mice made obese by the intraperitoneal injection of goldthioglucose had diaphragms that were as sensitive and responsive to insulin (range $3x10^{-4}$-10^{-2}U/mL)as were diaphragms from fasted lean mice. At 4-5 weeks of age,fasted ob/ob mice possessed diaphragms that were responsive to insulin at 3×10^{-3} and 10^{-2}U/ml.. but the response was still much less than for 8 week old lean mice.

Using a similar technique of injecting 1 ml. glucose-U-^{14}C in Gey and Gey bicarbonate buffer containing 3% albumin, Gershoff[50] also found that the diaphragms from ob/ob mice 3-4 months or 8-9 months old were totally unresponsive to 5×10^{-4} U/ml. of insulin, whereas 4-5 fold increments were obtained with diaphragms from lean mice.

A major difference between this *in vivo* bioassay and *in vitro* ones is the fact that the diaphragm is contracting in the *in vivo* bioassay. For example, it has been shown in this laboratory that in a perfused rat diaphragm muscle, contraction slightly increases the incorporation of glucose into glycogen in the absence of insulin, but in the presence of insulin the values do not differ significantly in the resting or contracting muscle[218]. Apart from the presence of factors released during exercise which increase glucose utilisation by muscle[216,219,220], the stimulated sympathetic nervous system would influence the glucose utilisation of muscle[222] indirectly by its effects on plasma free fatty acid concentration[221].

The reduced basal incorporation of glucose into glycogen seen in *ob*/*ob*-RD mice of 6-8 months of age is difficult to validly assess as, apart from their particular feeding regimen, which would be a very likely reason for the difference from fed *ad libitum* animals mentioned above, these mice had an obvious and exaggerated hyperactivity, presumably initiated by their hunger, and comprising a strong stimulus for the adaptation of a physiology of stress. However, the response of their quarterdiaphragms to concentrations of insulin above 5×10^{-4}U/ml was indistinguishable from that in lean mice at 6-8 months of age. In fact, these diaphragms were more sensitive to insulin, responding significantly to 5×10^{-5}U/ml. of insulin, at which concentration there was no significant effect in lean mice. Chlouverakis[55] has reported an exaggerated response to 10^{-3}U/ml. of insulin in fasted *ob*/*ob* mice, whose diet was acutely restricted, in both diaphragm and abdominal muscle *in vitro*; the response of the *ob*/*ob*-RD mice in the present work, on chronic restriction of food intake, was the same as that in lean mice. Fasting young

ob/ob mice for 24 hours prior to the experiments, when the glycogen content of their diaphragm is similar to that in lean mice[59], did not significantly increase the insulin effect on the isolated diaphragm, although it was found to reduce blood glucose and plasma insulin concentrations (Table 16, p.140). Fasting is known to leave unchanged[197] or to increase [86] the response of diaphragm muscle to insulin. The reduced responsiveness in diaphragm (and abdominal muscle) from overnight fasted ob/ob mice at 3-5 months of age has also been recorded by Chlouverakis[55].

The sensitivity towards insulin in quarter-diaphragms from lean and obese mice did not appear very different, both tissues responding maximally to a concentration of insulin of 5×10^{-4}U/ml. Wardlaw[114], although finding some daily variation, stated that the threshold insulin concentration in lean mouse hemidiaphragms was also in this range, viz. 10^{-4}U/ml. This is corroborated here, as 5×10^{-5}U/ml. was too low a concentration of insulin to consistently get an effect in quarterdiaphragms from lean mice.

These results suggest that the diaphragm muscle from an ob/ob mouse is sensitive to insulin but the magnitude of the response to the hormone is diminished. This is surprising because if the hyperinsulinaemia of ob/ob mice was a direct response to their hyperglycaemia, it can be seen here to be ineffectual in obtaining its disappearance, as raising the insulin concentration did not increase the magnitude of the response to the hormone in the diaphragm muscle from these mice. This is therefore an *in vitro*

manifestation of the tolerance to insulin observed *in vivo*. The hyperinsulinaemia of the older *ob/ob* mouse (6-8 months old) which one would expect to contribute towards the removal of glucose from the blood by increasing its capacity for uptake of glucose and synthesis of glycogen in muscle, does not appear to play this rôle in the diaphragm muscle from *ob/ob* mice. In the diaphragm muscle at least the hyperinsulinaemic response, which is probably not required in the more responsive diaphragm of the young *ob/ob* mouse, is ineffectual in increasing the capture of excess blood glucose by diaphragm muscle of 6-8 month old *ob/ob* mice.

Secondly, diaphragm muscle from older *ob/ob* mice ($>$ 6 months old) did not contribute to the hypothesised reduction in the uptake of glucose by peripheral tissues as there was no difference between lean and *ob/ob* mice in the incorporation of glucose-U-^{14}C into glycogen in the absence of insulin. *Ob/ob* mice in this age group had a stable hyperphagia, were inactive and were still hyperglycaemic. All these factors would normall be expected to lower the basal incorporation of glucose-U-^{14}C into glycogen (*v.s.* p. 161). However, *ob/ob* mice at 2-4 months of age were also hyperphagic and inactive and did have reduced basal glucose-U-^{14}C incorporation into glycogen. This suggests that the chronically elevated blood sugar level was the main determinant of the relatively raised basal glucose-U-^{14}C incorporation into glycogen in 6-8 month old *ob/ob* mice, compare to 2-4 month old *ob/ob* mice, and that this might have masked the reduced basal glucose-U-^{14}C incorporation into glycogen that probably persisted in *ob/ob* mice at 6-8 months of age. This view is substantiated by the reduced basal glucose-U-^{14}C incorporation into glycogen

in diaphragms from ob/ob-RD mice (on chronic food restriction), but not found when ob/ob mice were placed on acute restriction of food intake[55].

The significant sensitivity and responsiveness of quarterdiaphragms from fed 2-4 month old ob/ob mice, (not corroborated in fasted 3-5 month ob/ob mice by Chlouverakis[55] and Gershoff[50], or in 2 month old ob/ob mice by Stauffacher[59]), coupled with the remarkable response to insulin seen in quarterdiaphragms from ob/ob-RD mice, chronically or acutely[55] restricted in their food intake, suggests that the reduced insulin effect in the muscle of ob/ob mice is not a primary expression of the ob/ob genome but is probably an adaptation to the hyperglycaemia and developing hyperinsulinaemia. The fact that in young ob/ob mice, diaphragm muscle manifested both a reduced basal glucose incorporation and a reduction in the insulin effect at a high insulin concentration (5×10^{-3}U/ml.), (found *in vivo* in the plasma of these mice at this age), can be interpreted as some defect in the ability of this muscle to incorporate glucose into glycogen at a normal rate. It is not possible to say whether this was a reflection of their feeding state, or an adaptation to the increasing hyperinsulinaemia of the ob/ob mouse even at this young age. However, a reduced uptake of glucose by muscle with or without a reduced capacity to respond to insulin at the lipogenic phase of development of the ob/ob syndrome could be of great importance in shifting glucose uptake and utilisation to liver and adipose tissue.

Few studies have been made *in vitro* using the diaphragm muscle preparation in other genetic obesities or diabetes. No differences in the basal uptakes have been found in diabetic KK mice[223] or in the diabetic Chinese hamster[224],

although glucose oxidation was apparently lower in the muscle of these species[223,225]. As has been shown, the basal incorporation of glucose-U-^{14}C into glycogen is age-dependent which makes comparison with other studies difficult. Both the diabetic KK mice[223], the diabetic Wellesley hybrid mouse[226] and the obese sand rat[227], which are hyperinsulinaemic, also have diaphragm muscles that are insensitive to insulin.

B. Epididymal Fat Pads in vitro.

In common with early work on the subject[105], the oxidation of glucose-1-^{14}C in the absence of insulin was less than the insulin effect on this oxidation which was extremely variable, showing greater differences between animals than between pad fragments from the same animal[228]. Glucose-1-^{14}C oxidation was slightly but significantly raised in pads from 6-8 month old lean mice compared with the values at 2-4 months of age. Although glucose metabolism of adipose tissue is known to decrease markedly with age[229], no decrease was seen here because lean mice at 2-4 months of age have already achieve adult body weight and are sexually mature: the age differential in these experiments was too small and excluded very young immature mice.

In contrast to the work with diaphragm muscle, glucose metabolism in adipose tissue in vitro with 0.1% glucose in the medium (as measured by the oxidation of glucose-1-^{14}C) was markedly reduced in the tissue from ob/ob mice in both age groups, in agreement with the work of Christophe[56], Leboeuf[57], Chlouverakis[55] and Gershoff[50], (though in this latter study, incorporation into glycogen was increased in ob/ob mice 8-9

months old). This reduced glucose-1-^{14}C oxidation in ob/ob mice was threefold less than in lean mice in the 2-4 month old age group and fivefold less in the 6-8 month age group. This greater relative reduction in glucose metabolism in ob/ob adipose tissue with age has also been reported by others[50,130]. There was no absolute change in the glucose-1-^{14}C oxidation in pads from ob/ob mice with age, which suggests that, as the nitrogen content of the pads increased with age (Table 20) the reduced glucose metabolism was probably a maximal adaptation. A reduced basal metabolism of glucose has also been found in adipose tissue from diabetic mice (db/db) where it was found to decrease with age to below control levels[230]. Hypothalamic obese rats (VMN lesioned) have higher than normal basal values for glucose oxidation in adipose tissue[231].

The log dose-response range for the bioassay of insulin using the rat epididymal fat pad preparation in vitro is linear between 10^{-5} - 10^{-3}U/ml.[122-127,228]. Although rat adipose tissue is more responsive to insulin than adipose tissue from mice [232], a similar range was obtained in the present experiments except that there was a difference in the range of the bioassay for lean mice of 2-4 and 6-8 months of age. Mice of 2-4 months of age had pads that responded to insulin between 5×10^{-6} - 5×10^{-3}U/ml. while pads from mice of 6-8 months of age responded between 5×10^{-6} - 5×10^{-4}U/ml.

Epididymal fat pads from fed ob/ob mice appeared to be almost insensitive to insulin, especially in older mice[55,57]. Thus, concentrations as high as 5×10^{-1}U/ml. (1000 times the insulin concentration required to get a maximal insulin response

with tissue from lean mice) in the 6-8 month age group and 5×10^{-2}U/ml. (10 times the insulin concentration required to get a maximal insulin response with tissue from lean mice) in the 2-4 month age group were ineffective in obtaining a clearcut consistent response to the hormone. The average response of the tissue from ob/ob mice, as an increment in glucose-1-^{14}C oxidation, although significantly above zero, was never greater than 10% of the equivalent value for tissue from lean mice. These findings are contrary to those reported in the earliest study on the insulin sensitivity of adipose tissue from ob/ob mice, where Christophe et al.[56] found that the oxidation of glucose-1-^{14}C was reduced in the epididymal fat pad of 3-12 month old ob/ob mice but that in the presence of 10^{-1}U/ml. of insulin there was an approximately threefold increase in glucose-1-^{14}C oxidation. According to these authors, the dose-response of ob/ob adipose tissue to insulin was similar to that in lean mice. Although the experimental details were not identical to those reported here (viz. 0.2% gelatine was used as opposed to 4% albumin), the maximum effect on glucose uptake of 150% was recorded at a concentration of insulin 20 times greater than that needed (5×10^{-4}U/ml.) for a similar increment (as a percentage of the basal value) in the present work on glucose-1-^{14}C oxidation in pads from 2-4 months old ob/ob mice, (Fig. 12(a)). Gershoff[50] was unable to obtain an insulin effect on glucose-U-^{14}C incorporation into fat or glycogen in epididymal fat pads of 8-9 month old ob/ob mice with 10^{-4}U/ml. of insulin, a concentration which increased incorporation into glycogen 20 fold in lean mice. Insulin resistance *in vitro* in epididymal adipose tissue from ob/ob mice has also been found by Leboeuf[57].

The present experiments also contrasted with the report of Chlouverakis[55] who found 3-5 month old ob/ob mice as responsive as lean mice to 10^{-3}U/ml. of insulin when the increment in fat synthesis was calculated as a percentage of the basal value. As can be seen in Fig. 12 (a), although a +70% increase in glucose-1-^{14}C oxidation was obtained with 5 x 10^{-2} U/ml. of insulin in 2-4 month old ob/ob mice, this was three times less than the equivalent response obtained with adipose tissue from lean mice.

As in the results reported with diaphragm muscle on p. 100, fasting the ob/ob mice of the 2-4 month age group for 24 hours did not restore the responsiveness towards insulin in the epididymal fat pad[55] nor were the basal values of glucose-1-^{14}C oxidation to $^{14}CO_2$ increased (Fig. 10(a)). Fasting is known to reduce the response to insulin in adipose tissue in rats[86,136] so one would not expect this treatment to eradicate the insensitivity found in tissue from ob/ob mice.

Weight reduction is known to increase the sensitivity and response of rat adipose tissue to insulin[234]. However, although the adipose tissue from ob/ob-RD mice on chronic restriction of food intake oxidised glucose-1-^{14}C at a normal rate, unlike the experiments with diaphragm muscle, th response and sensitivity to insulin were only partially restored. Chlouverakis[55], who had studied ob/ob mice on acute restriction of food intake (see p. 13), found higher basal lipogenesis from glucose in these mice. The effect of 10^{-3}U/ml. of insulin was also much greater than in lean mice These very contrasting results could very well be due to the different metabolic and endocrine status of ob/ob mice on acute and chronic restriction of food intake.

In parallel with experiments on diaphragm tissue, Stauffacher et al.[59] using the in vivo intraperitoneal injection technique referred to above, found that the labelling of epididymal adipose tissue lipids from glucose-U-^{14}C in 8 week old ob/ob mice increased significantly only in the presence of 10^{-2}U/ml. of insulin, whereas tissue from lean mice of the same age responded at 3×10^{-4}U/ml. At 5 weeks of age the pads from ob/ob mice were as sensitive to 3×10^{-3}U/ml. of insulin as pads from lean mice; (the labelling of lipid, expressed as CPM/fat-free dry weight in the absence of insulin, was higher in ob/ob mice, both at 5 weeks and at 8 weeks of age). These results are difficult to compare with the present in vitro work (see also discussion on p. 165). but the poor responsiveness of ob/ob adipose tissue from the second month onwards was confirmed. Using a slightly different medium for injection, Gershoff[50] found 8-9 month old ob/ob mice unresponsive to the intraperitoneal injection of 5×10^{-4}U/ml. of insulin as measured by the incorporation into glycogen. At 3-4 months of age, although the response in fat synthesis was still much less than was found in lean mice, the response in glycogen synthesis was no different to that found in lean mice. Further, the basal incorporation of counts into either adipose tissue glycogen or lipid was the same in both ob/ob and lean mice at 8-9 months of age and was different only in the incorporation into glycogen at 3-4 months of age. Gershoff[50] however, found that when incubated in vitro, fat pads from 8-9 month old ob/ob mice were relatively unresponsive to 10^{-4}U/ml. of insulin either in an effect on glycogen or on fat synthesis.

The data obtained here which suggests that the adipose tissue of ob/ob mice is unresponsive to insulin after about three months of age is thus in broad agreement with much of the related work in adipose tissue from ob/ob mice.

The age of the animal has a much more dramatic effect in the pad preparation[50,56,233] than was seen with diaphragm muscle. Adipose tissue from lean mice of 2-4 months of age showed an increasing response to insulin until a concentration of 5×10^{-3}U/ml. was reached, and therefore responded to a greater extent than tissue from lean mice of 6-8 months of age whose maximal response occurred at 5×10^{-4}U/ml. The sensitivit of both tissues was the same, a response being detectable with as little as 5×10^{-5}U/ml. of insulin.

Reduced responsiveness to insulin in fat pads has been found in other genetic obesities with hyperinsulinism, viz. in the New Zealand obese mouse[59], the diabetic KK mouse[223], the diabetic C_3Hf 1 Wellesley hybrid mouse[226], the diabetic db/db mouse[230] and the obese sand rat, Psammomys obesus[227,235]. This insulin resistance was not found *in vitro*[56,57] or by intraperitoneal bioassay[59] in the fasted goldthioglucose-obese mouse, despite the hyperinsulinaemia[48,258] and *in vivo* resistance to the hypoglycaemic action of insulin of these mice[236], (not found by Mayer[237]). Further, adipose tissue from these mice[238] and from hyperinsulinaemic[239,240] VMN-lesioned rats that became obese[241], responded subnormally to epinephrine. The occurrence of reduction in the responsiveness to insulin in other genetic obesities in mice and in human obesity[80], (where it is dependent on caloric intake[80]), again suggests perhaps the universality of this feature in different obesities.

C. Isolated Adipocytes in vitro.

The experiments on the sensitivity of isolated adipocytes to insulin were carried out for two reasons: firstly, in order to utilise a sensitive and more accurate insulin assay[136] and secondly, to compare the insulin sensitivity in cells from two morphologically dissimilar preparations and hence to investigate the role played by extra-adipocyte factors and tissue morphology in insulin sensitivity and response.

The increased sensitivity of isolated adipocytes compared to that of fat pads is evident in the results presented for both lean, ob/ob and ob/ob-RD mice. Young 2-4 month old lean mice had adipocytes that were responsive to insulin in the range 5×10^{-7} to 5×10^{-5} U/ml. (medium glucose concentration 0.03%) and 5×10^{-6} to 5×10^{-4} U/ml. (medium glucose concentratio 0.2%) as has been found for rat adipocytes[92,93,136,242]. For an equivalent medium glucose concentration, the insulin effect recorded here, as a percentage increment in the glucose-1-^{14}C oxidation in mouse adipocytes, was in good agreement with that obtained by Gliemann[92,93] and Goldrick[242]. The percentage increment with insulin, however, was slightly smaller in adipocyte suspensions compared to that obtained in pads from 2-4 month old lean mice. The reason for this was probably associated with the preparative procedure for adipocytes during which time some damage to the insulin "receptors" on the adipocyte surface could have occurred[93]. The results with adipocytes from ob/ob mice in particular, were in marked contrast with the equivalent findings in fat pads incubated in vitro. Cells from 2-4 month old ob/ob mice were markedly responsive to low concentrations of insulin (5×10^{-5} U/ml.) at both glucose

concentrations although these insulin concentrations were slightly higher than those required by cells from lean mice. Fat cells from older lean mice were relatively insensitive to insulin in the presence of 0.2% glucose although, as found with adipocytes from VMN-lesioned mice that became obese, they were sensitive at the lower glucose concentration. These results were in keeping with a reduction in sensitivity towards insulin with age found in the epididymal fat pad experiments. Again, cells from ob/ob mice of 6-8 months of age although less sensitive than at the younger age group, were sensitive to insulin at both glucose concentrations.

In marked contrast to the findings with fat pad, the effect of keeping the ob/ob mice on a restricted diet was to significantly increase the insulin sensitivity and responsiveness of the adipocytes as compared with those from lean mice. Similar findings have been obtained in incubated pads from ob/ob mice on acute dietary restriction (see p. 13)[55] and in adipose tissue fragments from obese humans after weight reduction[80]. This suggested that the impaired sensitivity of isolated adipocytes from fed ob/ob mice was not a primary defect in the ob/ob syndrome for it could be altered by a change of food intake and feeding regimen; hyperphagia might have been modifying the hormonal effect. The impaired sensitivity was more likely to have been an adaptation to the hyperinsulinaemia and hyperglycaemia of the ob/ob mice, one manifestation of which was the persisting response to a high concentration of insulin (5×10^{-3} - 5×10^{-2} U/ml.) in the presence of a high medium glucose concentration in 6-8 month old mice. The range of the dose-response at this age had therefore shifted to a range

where, at higher plasma insulin and glucose concentrations, it was probably still effective (in the absence of other controlling factors in the intact tissue or in its blood flow *in vivo*). Thus, insensitivity of isolated adipocytes to insulin is not a primary defect in *ob/ob* mice.

The differences found in the insulin sensitivity of the fat pad and of the adipocytes of both the *ob/ob* mice and the *ob/ob*-RD mice has at least three possible explanations. Firstly, probably due to the fragility of the large cells from the obese mice pads, which were, therefore absent from the suspensions prepared from these pads (Fig 16), the difference in insulin sensitivity in fat pads and in adipocytes could be explained by the hypothesis that insulin sensitivity is related inversely to adipocyte size[80]. Secondly, the *ob/ob* adipose tissue may have contained some factor which inhibited or interfered with insulin activity or had endogenous insulin-like activity and this factor was lost in the preparation of fat cells. For example, it has been shown that the cells of the reticulo-endothelial system, in particular the mast cells, are more numerous in the adipose tissue from *ob/ob* mice than in that from lean mice[31] and one could speculate that these cells were in some way interfering with the insulin effect. Thus the adipose tissue preparation which contained only fat cells would have retained its insulin sensitivity. Alternatively, factors which reduce the apparent insulin effect (which may be hormones[88] or endogenous insulin[90]), might have originated from the blood retained in the interstices of the tissue matrix and were not removed by the preparative washing of the pads before incubation. The endogenous insulin in particular, which was high in *ob/ob* mouse serum, might have reduced the insulin effect on *ob/ob*

pads whether the endogenous insulin was bound to the tissue or was free in the retained serum. Thirdly, the reduced insulin effect in the fat pads could have been due to a lack of penetration of the hormone which might have prevented the added exogenous insulin from reaching all the active cell receptors in the fat pads of the ob/ob mice. This could have been caused by a defect in the basement membrane of insulin-sensitive tissues in ob/ob mice or by an increased binding of insulin to peripheral capillaries[243]. Chapter III F17 presented evidence showing that the epididymal adipose tissue of ob/ob mice at 2-4 months of age had fewer and larger fat cells and a lower water content (per unit wet weight of tissue) than was found in lean mice. The reduced ^{125}I-albumin space in the pad from ob/ob mice (per unit wet weight of tissue) favours the suggestion that there was a reduced intravascular fluid space (per unit wet weight) in the pad from ob/ob mice. However, attempts to measure the extracellular fluid space with ^{3}H-Sorbitol were unsuccessful (see p. 189). Reduced extracellular fluid space in ob/ob pads is still unlikely as an explanation of reduced pad responsiveness to insulin in ob/ob mice as a similar insensitivity and reduced responsiveness of epididymal fat pads from 8 week old ob/ob mice had been shown by a completely different *in vivo* intraperitoneal insulin bioassay[59] which did not rely on adequate diffusion of substrate or hormone from an *in vitro* incubation medium. Further, three hour incubations of pads from ob/ob mice, (when the effects of a possible difference in ^{3}H-sorbitol equilibration during the first hour would be greatly reduced (see p. 189)) still show reduced glucose metabolism[57] and

insulin sensitivity[57].

This is the first detailed report of the insulin sensitivit of adipocytes in an animal that is genetically obese. In a preliminary study, Stauffacher et al. also found ob/ob adipocytes almost fully responsive (glucose concentration 0.09%) to 10^{-3}U/ml. of insulin and with a normal basal glucose metabolism related to DNA content of the suspension[244]. However, as a dose-response curve was not obtained, no conclusions as to sensitivity could be made. Genetically obese rats or VMN-lesioned rats that became obese possessed adipocytes that were unresponsive to an insulin concentration of 10^{-4}U/ml. with a medium glucose concentration of 0.1%[245], in contrast to the results obtained in Table 15 with VMN-lesioned mice that became obese.

D. In vivo Levels of Blood Glucose and Plasma IRI

The plasma IRI of ob/ob mice fed ad libitum has been shown to be considerably higher than concentrations found in fed lean mice even when their blood glucose was raised to over 300 mg.% by intragastric glucose incubations. Further, the plasma IRI of ob/ob mice did not fall to levels found in lean mice after 6 or 24 hours fasting in contrast to the results of Stauffacher[48] although blood glucose values, still high after 6 hours fasting, were not elevated after 24 hours fasting. Plasma IRI concentrations are also raised in maturity-onset diabetes both after overnight fasting and postprandially[66a,66b 67,70-72ab]. The concentrations of plasma IRI in lean mice after various glucose loads were also much higher than previously published values for mice[46,48]. The high values reported in this study were probably due to the fact that an assumed linear

biological scale had been used to evaluate a relatively flat immunoassay curve produced with antibody and ^{125}I-insulin from another species[246].

The assay of immunoreactive insulin is highly specific for insulin and is considered by most workers to represent the active circulating form of insulin[66b]. Although doubt has been expressed as to the validity of comparing the immunologically active site of insulin with its biologically active site[124], there are some published reports showing close correspondence between the two[48,247] and much direct evidence which suggests that for some antibodies similar parts of the insulin molecule are required for full biological and immunological activity[248,249]. Obese mouse serum has been subjected to simultaneous biological and immunological assay by Stauffacher[48] and a high degree of correspondence between the two was obtained, confirming the high biological activity of ob/ob mouse plasma first reported by Christophe et al.[51]. Although these reports do not conclusively demonstrate the absence of a biologically inactive insulin in physiological conditions or under conditions which circumvent the criticism that biologically active insulin is formed in the bioassay procedure from an originally inactive form, it is reasonable to presume that the insulin levels recorded in the present work represent a biologically active form of the hormone.

Although other metameters of intermediary metabolism (e.g. liver glycogen) may be explained as responses to the concomitant blood glucose concentration (see Table 17), our findings suggest that this may not necessarily be true for the hyperinsulinaemia of obese-hyperglycaemic mice. Ob/ob mice manufactured more

plasma IRI in response to a glucose stimulus, whether of subcutaneous or intragastric origin, even when their blood glucose levels were not particularly elevated. The dependence of the high plasma IRI concentrations on recent administration of glucose was shown by:

(i) the enormous concentrations of plasma IRI after administering intragastric glucose repeatedly (3 times) in fed ob/ob mice, and

(ii) the rapid/sustained rise in plasma IRI concentration after intragastric or subcutaneous glucose loads in 24 hour fasted ob/ob mice.

This increase in pancreatic secretory capabilities (which is deduced from the fact that no impaired destruction of circulating IRI in ob/ob mice has been shown[52]) was further strongly stimulated by continued glucose loading; no such phenomenon existed with lean mice even with blood glucose levels of over 300 mg./100 ml. These experiments showed that the hyperinsulinaemia of obese mice depended on an initial glucose stimulus whether of endogenous or exogenous origin, and that the pancreatic β-cells of these mice (at least by 6 months of age) were either normally sensitive but subject to an excessive insulinogenic drive other than blood glucose or were in a state of high intrinsic sensitivity to stimulation by known insulinogenic stimuli[137,138,250-255] especially circulating glucose. The fact that in ob/ob mice which received large amounts of intragastric glucose, IRI and glucose levels were not closely correlated, as well as the increased synthesis of IRI in the presence of unelevated levels of blood glucose in the post-starvation refed state, suggest that stimuli other

than
/glucose may be important. It seems unlikely that only intestinal insulinogenic factors are involved, as subcutaneous injections of glucose were also effective in producing the hyperinsulinaemia of ob/ob mice. Further, the continued secretion of increased amounts of IRI 6 or more hours after the last feed also argues against this possibility. The brisk postprandial plasma IRI response in normal human subjects is absent in the adult diabetic state[66b]. Further, there is a delayed secretion of plasma IRI after glucose loading in such subjects[256]. It is possible that such a phenomenon also occurred in ob/ob mice; it must be emphasised that the elevated plasma IRI values found in ob/ob mice are of a completely different order of magnitude to those found in obese diabetics or obese non-diabetics clinically[66b]. Plasma IRI is also elevated following intravenous glucose in obese non-diabetic patients with normal glucose tolerance tests[67,70-72ab], and this hyperinsulinaemia is absent after correction of the obesity[78-80].

Ob/ob-RD mice which were fed a restricted diet, invariably entirely consumed in a short period of time, have normal blood sugar levels (Table 16) and are known to respond normally to the hypoglycaemic action of exogenous insulin in vivo[54,55]. The presence of high plasma insulin concentrations in these mice (with only residual hypertrophy of the pancreas[257]) and in 24 hour fasted ob/ob mice suggests that the hyperinsulinaemia of these animals may closely reflect their primary defect, and probably explains the persistence of excessive fat synthesis in "restricted diet" animals[83ab]. However, the partial or nearly complete disappearance of the excessive plasma IRI

response to glucose in fasted young ob/ob or in 6-8 month old ob/ob-RD mice, both of which do not show the hyperplasia of the islets of Langerhans typical of the adult fed ad libitum ob/ob mouse[46,257], as well as the presence of high concentrations of blood IRI in obesity caused by hypothalamic damage[48,231,239,240,258-260], and the fact that in the early phase of genetic obesity pancreatic insulin content is less than in lean mice[33a,46,48], could all suggest that the primary defect may be at a locus other than (or additional to) the process of pancreatic β-cell response to glucose.

Hyperinsulinism has now been established as a characteristic of the ob/ob mouse and of experimental and genetic obesities in animals whether the animals are hyperglycaemic or not[48,231,239,240,258-268]. The only exception to this so far is the diabetic Chinese hamster which suffers from an insulinoprivic diabetes[269]. Restriction of food intake also reduces the plasma IRI concentrations in diabetic KK mice[223] and sand rats[270]. It has no effect on the plasma IRI of spiny mice (Acomys cahirinus) even when they are not hyperglycaemic[268], and only succeeds in stabilising the high levels of plasma IRI (with no effect on the pancreatic insulin content) in the db/db mice[230].

E. Cellular Characteristics of Intact Epididymal Fat Pads

Some investigators have reported that obesity in the human[271,272], the rat[273] and the mouse[35,131] was associated with a decrease in the percentage of water and an increase in

the percentage of lipid in adipose tissue; the percentage of protein either stayed the same[272,274] or was reduced[210,273]. Other reports on the composition of human[274] and mouse[210] abdominal adipose tissue in obesity and in the hypertrophy of the adipose tissue of rats made obese by repeated injections of insulin[275] have shown no difference in composition from that found in lean controls.

In this study no significant difference in the nitrogen content (per unit wet weight) was observed between pads from obese and lean mice, either at 2-4 months of age or at 6-8 months of age. As pads from obese mice are 2-9 times heavier than those from lean mice, the total protein per pad from obese mice is considerably higher than that from lean mice[56,210]. Assuming that an increase in adipocyte size occurs largely by increase in the lipid content of the cell[276], then the fact that there was no change in the amount of nitrogen per unit weight in the ob/ob pads could be explained either by an increase in adipocyte number large enough to mask an increase in adipocyte size, or by increases in structural stromal proteins or cell types other than adipocytes[31,132], or lastly, but less probably, by an increase in the nitrogen content of the excess lipid stored.

In order to determine whether the increase in size of the epididymal fat pad was achieved by an increase in the size and/or number of its adipocytes, adipocytes from pads of 2-4 month old lean and obese mice were counted and sized by the method of Hirsch et al.[180a]. Table 20 shows that the number of adipocytes per unit wet weight of this tissue was nearly 4 fold greater in the pads from lean mice and that they

were much smaller in size (Fig. 16) than those seen in ob/ob pads. The increase in the size of adipocytes from fat pads of 2-4 month old ob/ob mice, shown in Fig. 16, has also been determined by histological examination of fixed, stained sections of tissue[31,132] and in isolated cell suspensions[277]. Hellman[31] has argued that the increased fat content of the adipose tissue from obese mice occurred by simple enlargement of existing cells, mature adipose cells being non-mitotic[278], a hypothesis that has been advocated for other obesities by some investigators[279,280]. Assuming a 6-8 fold increase in pad wet weight in obese mice at this young age[56,130], it is clear that the number of adipocytes in the epididymal pads of these mice could not have increased by more than a factor of 2 in these young mice. This interpretation has been drawn from experiments on epididymal fat pads from young ob/ob mice and may not be true of the more pronounced hypertrophy and hyperplasia in the subcutaneous adipose tissue in older mice which Hausberger[132] has found to be increased in thickness because of an increase in cell number and size, (although some fat cells in sections from this subcutaneous adipose tissue were found to be of normal or small size (40 μ or less)). Sizing and counting of adipocytes in sections of adipose tissue is open to error as a section does not pass through the centre of all the cells in a sample. Thus, these results are in keeping with other forms of clinical and experimental obesity where the excess fat content of adipose tissue was found as an increase in the size and number of the adipocytes[273,279,281-283] although an increase in DNA per pad was often probably falsely equated with an increase in adipocyte number[273,281-282]. It is well established that adipocytes in

normal rat epididymal fat pad contribute less than a third to the total DNA of the tissue[280,284], and therefore conclusions on the cellularity of adipose tissue based on the DNA content of the tissue must be suspect as adipose tissue is invaded with other cell types when chronically enlarged[132].

In studies on obesity induced with goldthioglucose in 8 week old mice, Liebelt[120] suggested that adipose tissue enlarged by an increase in adipocyte number only after the existing cells had reached a "saturation" level of a certain ideal size with a maximum lipid:fat-free dry residue ratio repre-sentative of the maximum fat cell size attainable for that particular species. However, as explained previously, the fat-free dry residue of a fat depot cannot be equated with adipocyte number. Thus it has been shown by Hirsch[280] that there was no further increase in adipocyte number per epididymal fat pad after the 12th week in normal rats, and, in rats made obese by hypothalamic lesions, enlargement of the fat pad was entirely by an increase in adipocyte size. He has suggested that as adipose tissue aged, it progressively lost the ability to grow by hyperplasia of its adipocytes, perhaps because their rate of formation was equal to their rate of death. It is clear that data on tissues from obesities of varying severity and age and from different anatomical locations differing in stromal protein content, and using protein or fat-free dry residue values that may not attest to the actual increase in the number of adipocytes present, make comparison with other published reports difficult to interpret.

The data presented here, which suggests that epididymal fat pads from obese mice have a maximum increase in adipocyte number of 2 and a 4-fold decrease in the number of

adipocytes per unit wet weight of tissue, agrees well with metabolic data on the oxidation of glucose-1-^{14}C by this tissue if it is assumed that the unit of this metabolic activity in this tissue is the adipocyte. It has been shown[80] that glucose metabolism in human adipose tissue fragments was dependent on the number and not the size of the adipocytes in the tissue. This suggests that the reduction (per unit wet weight) in the *in vitro* glucose-1-^{14}C oxidation (Fig. 10 (a) and (b)) and ^{14}C-acetate metabolism[31] in the incubated pads of lean and *ob/ob* mice, and in the *in vivo* data on the uptake of radioactive glucose and acetate into carcass lipids or epididymal fat pad tissue of *ob/ob* mice[130,285,286], appears proportional when related to the reduced number of adipocytes per unit wet weight found in this tissue.

Weight reduction by a restriction of the caloric intake in human[287] and rat adiposity[273,288] has been shown to increase the percentage of fat-free residue and water respectively, together with the DNA per pad, while the percentag of lipid decreased. There is much evidence showing that there is no loss of cell number in adipose tissue during acute starvation (when the pad weight may approach 10% of its original weight) in the human[287], lean rats[289,290], obese rats[273], or rats[280] or obese mice[132] maintained on a restricted diet for 3-5 months. The unelevated weights of the epididymal fat pads from acutely starved (11 days) obese mice were not associated with a significant reduction of the mean adipocyte size in epididymal adipose tissue[132], (although great reduction occurred in subcutaneous adipose tissue[134]). As the weight reduction of the epididymal fat pads appeared to have occurred

by mobilisation of fat from small fat cells, reducing their size still further; this probably resulted in their not being recognized as fat cells. These findings were not therefore inconsistent with there being no loss of adipocyte number in this tissue. The significant reduction in the nitrogen content per unit wet weight of epididymal pads from these mice (Table 20) could therefore be explained by a reduction either in non-adipocyte cell types, stromal elements or nitrogen containin lipid. The ready release of fat from this tissue, which would literally swim in its own fat, also suggested that the lower nitrogen content of this tissue may be related to a release of intact protein known to occur in adipocytes and fat pads in whom lipolysis has been stimulated[291].

The plasma volume of 1.49 ml. for lean mice is in agreement with some published data on the plasma volume of mice by other methods[131,292-295]. For the dilution principle to be valid, the circulatory system must have no leak; this condition was difficult to stringently satisfy as the abdomen had to be opened (the portal vein being the only vein not completely encased in fat, and thus accessible) and tracer was injected into very small veins. However, the plasma volume of obese mice was found to be slightly but significantly higher than that found in lean mice, and the increase, similar to that found by Yen[295], but not by Mayer[131], was consistent with the small 10-20% increase in the body water content of these mice.

The decreased water content[131] and plasma volume as a percentage of the carcass weight was reflected, in the ob/ob mouse, by a reduction in the water content and *in vivo* ^{125}I-albumin space per unit wet weight in the epididymal fat pad. Most of the water content of the pad reflected water in

cells other than in adipocytes and extracellular fluid. This was because the water content of adipocytes is very small (3-6 μl/100 mg.)[296]. The *in vivo* labelling of the vascular compartment contrasted clearly with the *in vitro* distribution of ^{3}H-sorbitol under conditions identical to those used in the insulin sensitivity work. No statistical difference between lean and obese mice was observed in the ^{3}H-sorbitol space when pads were incubated *in vitro* for 60 minutes. An increase in the stromal elements of adipose tissue in *ob/ob* mice could be expected to slow the rate of entry of the ^{3}H-sorbitol into the tissue[297]. In this determination, no attempt was made to measure the rate at which equilibrium was reached so that it was not known if the ^{3}H-sorbitol had fully equilibrated with the extracellular tissue space after 60 minutes in pads from both lean and *ob/ob* mice. In rat epididymal fat pads, a doubling of the incubation period leads to a 20% increase of the extracellular ^{3}H-sorbitol space and water content of the pad[297], (and the water content of pads incubated for 60 minutes was 20% higher than in non-incubated tissue[297]). The increase in the extracellular space during an incubation procedure[297] and the possible differential in the rates of ^{3}H-sorbitol equilibration with the extracellular space made this data difficult to interpret correctly.

The surprising decrease in the median cell size found in *ob/ob* adipocyte suspensions compared to that found in the intact tissue, has a number of possible explanations. The most likely reason is due to the fact that direct fixing with OsO_4 of collagenase-prepared adipocytes at room temperature for between 3 and 24 hours did not alter their spherical shape as

it did when intact pads were fixed with OsO_4 for much longer periods of time. Although it sometimes was possible to distinguish adipocytes from fat droplets by the degree of staining under the microscope this was variable and inaccurately reflected in the photomicrographs. As ob/ob adipocyte suspensions varied considerably in the lysis that occurred during their preparation (Table 14), it was probable that considerable lysis had occurred in the 3 suspensions studied here and the distribution profile shown in Fig. 16 is suggestive of a major proportion of fat droplets from lysed cells in these preparations.

The same was probably also true of the adipocyte suspensions from lean mice but this has clearly occurred to a lesser extent. It was however possible that the distribution profile of ob/ob adipocyte suspensions reflected the existence of large numbers of immature adipocytes. They have been commented upon by Hausberger[132] who examined sections of subcutaneous and abdominal adipose tissue from these mice. These may have been present in the photomicrographs of adipocytes from cell suspensions as these suspensions were not filtered through a mesh (30μ pore size) before their fixation, whereas this step was included in the Hirsch procedure[180a].

CHAPTER V

CONCLUSION

1. GENERAL CONSIDERATIONS ON COMPARING OB/OB TO LEAN MICE.

The study of obesity has at its centre a problem which is fundamental to data comprehension and which often eclipses the philosophical concepts of science based on the dictum that "all science is measurement" (Helmholtz), because in the words of Sir Henry Dale, "all true measurement is essentially comparative". It is thus, in the final analysis, a question of agreed opinion as to how one compares a fat, inactive, hyperphagic ob/ob mouse with its lean littermate. The problem becomes more difficult in a type of genetic obesity in mice where lean littermates do not exist.

(a) Quantitative expression of data.

Data expressed per total weight of ob/ob mouse, or per mg. wet weight of adipose tissue of these animals, is bound to show a reduction compared to lean mice. This is because, the excess weight of both carcass and adipose tissue being largely composed of fat, most parameters, e.g. the perfused vascular space, water content and number of cells per mg. epididymal adipose tissue and the plasma volume, oxygen consumption[298], BMR[209] and water content[131] of the whole animal, are greatly reduced in the ob/ob mouse.

(b) Quantitative administration of hormones/substrates.

Drugs, hormones and substrates administered intraperitoneally or intravenously to ob/ob mice have usually been given in doses proportional to the body weight and not in proportion to the lean body mass. It has been shown in ob/ob mice that it is not correct to compare the efficiencies of the glucose homoeostatic mechanisms with a glucose load dependent on the total body weight[33b]; even lean mice show a glucose intolerance when presented with a very large glucose load (Table 17). Also it is not correct to administer the same amount of a test substance to both ob/ob and lean mice, as the plasma volume of ob/ob mice is slightly but significantly larger than that of lean mice (Table 20). The expression of data when comparing relatively fat-soluble substances with water-soluble substances in ob/ob and lean mice emphasizes this difficulty, as the volume in which fat-soluble substances would be distributed would be much larger in ob/ob mice.

(c) Effect of differences in voluntary activity.

Ingle[299] has described the difficulties associated with the study of the endocrine influences on metabolism and those which are particularly evident when looking for common parameters with which to express metabolic disorders. He mentions the genetic component of voluntary activity, and the fact that voluntary activity is reduced to a very low level by castration, adrenalectomy, hypophysectomy or thyroidectomy. Also, the restriction of the voluntary activity of a rat can lead to obesity. This probably derives from the fact that there is a very marked effect of muscle stimulation upon glucose tolerance in the eviscerated rat with and without insulin[220]

and this may be due to a circulating hormonal factor[216,219]. Thus muscular activity may be responsible for metabolic differences or alternatively, endocrine deficiencies and excesses are known to modify voluntary activity.

(d) Effect of hyperphagia.

The hyperphagia of ob/ob mice may also control their metabolic and endocrine responsiveness, (see p. 21) for discussion of ob/ob-RD mice), by increasing the number of feeding periods each day[300a] as well as by an alteration in food intake per se. Goldthioglucose obese mice[48,258] and hypothalamic-obese rats[239,240,259] which are known to be hyperphagic[142,300b], are hyperinsulinaemic with high levels of IRI in their pancreas. Their adipose tissue and diaphragm in vitro are not insulin resistant when these mice are studied in the fasted state[56,57,59], but in vivo they are resistant to the hypoglycaemic action of insulin[236], (although Mayer[237] finds no such abnormality). Control of the hyperphagia found in many genetic obesities by chronic or acute restriction of food intake results in the amelioration of many of the abnormalities in these syndromes.

(e) Effect of the selective absorption of dietary factors.

An adequate supply and absorption of vitamins from the diet is needed for full responsiveness to hormones. This has been well illustrated by the requirement of folic acid for the biologic response to oestrogens and of pyridoxine for the response to androgens[301]. Dietary factors necessary for full responsiveness to insulin have not yet been reported.

Until this has been further studied, however, it remains a possible important determinant of insulin activity.

Muscle activity and the selective intestinal absorption of dietary factors have been ignored in nearly all studies on the aetiology of the obesity of the ob/ob mouse. The use of the ob/ob mouse on a restricted diet, although introducing other variables, has provided much information on the role of hyperphagia in the obese hyperglycaemic syndrome. In this connection, mention should be made of the use of goldthioglucose obese mice or rats or mice made obese by stereotaxically placed electrolytic lesions in the hypothalamus in order to minimise the differences in the morphology of adipose tissue (e.g. to avoid differences in size of fat cells) and in voluntary activity found in the genetically obese animal and in its lean littermate[59,245]. It is clear that, of the many differences found between lean and ob/ob mice, many will be due to the inefficient controlling of these variables and only a few will be due to the genetic defect.

2. ROLE OF INSULIN RESISTANCE IN THE OBESE-HYPERGLYCAEMIC SYNDROME WITH SPECIAL REFERENCE TO THE OB/OB-RD MOUSE.

The clear responses to insulin of the isolated diaphragm muscle and adipocyte suspension from ob/ob-RD mice and of the adipocytes from ob/ob mice fed ad libitum demonstrated in this work, suggest that a decreased sensitivity to insulin at the cellular level is not the primary defect in the obese-hyperglycaemic syndrome, but is probably an adaptation to the hyperinsulinaemic hyperglycaemic state of these animals. This conclusion is supported by the work of other investigators[55].

It is evident that the caloric value of the diet and/or the feeding pattern can influence, either directly[302] or indirectly by alterations in adipose tissue blood flow[303], the sensitivity and responsiveness to insulin in ob/ob mice. Experiments on rats have also shown that restricted periods of food intake increase the hypoglycaemic effect of insulin in vivo[304,305] and also produce elevated insulin concentrations in the pancreas[306]. In the present work, the ob/ob-RD mice, which received 5 g. of food a day, an amount equal to or less than the normal food intake of lean mice[140], were still obese (see Plate 1). It is of interest in this connection that rats fed for a short period once a day become obese and lipogenesis in the adipose tissue[307] and liver[307,308] of these animals is increased. Also, a return of the responsiveness to insulin of peripheral tissues after diet restriction has been recorded for the diabetic KK mouse[223] and the diabetic C_3HfI Wellesley hybrid mouse[226], but the resistance together with a reduced basal glucose metabolism persisted in the adipose tissue from db/db mice[230] on this diet.

metabolic and endocrinological consequences of undernutrition and the resultant alterations in feeding habits are numerous and have complex ramifications[304]. For this reason it is difficult to know whether the adaptation of tissue responsivenes to insulin in ob/ob-RD mice is revealing a defect due to the ob/ob gene or is in fact masking such a defect. The ob/ob-RD mice, however, have served to illuminate features of the syndrome that persist when most of the characteristic features of the fed ad libitum animals, such as the hyperglycaemia, the increased tolerance to insulin in vivo, the lack of insulin responsiveness in adipose tissue and muscle in vitro, and the infertility, have disappeared.

Physiological role of the hypothalamus and pituitary somatotrophic factors in obesity.

The association of obesity with hypothalamic disease was noted as early as 1840[309]. Further, diabetes and diabetes with obesity have been observed in patients with cerebral tumours [310], and in one case of tuberculous meningitis[236]. Individual instances of diabetes mellitus produced by direct hypothalamic injury have also been recorded [141,236,311-313].

The possibility that the pituitary controls responsiveness to insulin has been suggested by in vivo[314] and in vitro[315] studies and there is evidence that it influences the plasma insulin-like activity[316]. Another possibility therefore for the effect of restricted diet on the insulin response in ob/ob mice is that the hypothalamus or the pituitary may be involved. It has been shown that the infertility observed in the ob/ob mice fed ad libitum is due to

deficient gonadotrophin[317-319] in these animals and that fertility is restored by food restriction[82]. This suggests that food restriction must affect in some way the hypothalamus or the pituitary. Differences in the histology of the pituitary have not been observed in ob/ob mice[320], or in the diabetic KK mouse[321].

Mayer[322] first postulated the hyposecretion of somatotrophic pituitary hormones as a possible cause for the obesity in ob/ob mice. However, a search for hypothyroidism in these mice was negative, the iodine uptake being normal with no abnormality detectable in thyroid histology[209]; also the administration of thiouracil does not mimic the ob/ob syndrome. Similar negative effects of goldthioglucose on thyroid function have been reported[323]. However, prolonged triiodothyronine (T3) administration to ob/ob mice, eliminates the hyperglycaemia and increases the sensitivity to insulin in vivo[209]; ob/ob mice are known to be unusually sensitive to thyroxine[209].

The normal response to fasting of plasma FFA levels in ob/ob mice[324], suggests the existence of an active pituitary, for it has been shown that the latter is necessary for the full response to occur in rats[325]. Hypophysectomy is known to result in obesity (but without hyperglycaemia) in chickens[326]. It would be interesting to discover whether ob/ob mice possess a genetic mutation in the hypothalamus or pituitary that has a phylogenetic history. The fact that a mouse with a hypothalamus damaged by goldthioglucose exhibits hyperglycaemia when it is exposed to non-specific "stress" stimuli viz. cold or the daily injection of cortisone, would suggest that the normal hypothalamus probably has an important inhibiting action perhaps

by raising the threshold for its response to the "feedback" hormones so as to lead to the production of hyperglycaemia and glycosuria[236] - (for further discussion of this point see Chapter V, p. 205).Also ob/ob mice are abnormally sensitive to hormones such as growth hormone[327] and thyroxine[209] which have a feedback inhibitory action on the pituitary,and this favours Mayer's speculation of low or absent secretion of pituitary somatotrophic hormones which are not activated by short-term neural "stress" circuits, (e.g exposure to cold), but are activated by long-term stress due to food deprivation, (e.g. ob/ob-RD mice). (Ob/ob mice can survive up to 17 days without food (viz. when stereotaxically-lesioned in the lateral nucleus of the hypothalamus)). Again short-term glucose deprivation as in the 24 hour fast does not affect the sensitivity and response to insulin presumably because the physiological consequences of 24 hour fasting do not allow the same time for adaptive effects on the structure and composition of cellular enzymatic machinery to occur.

3. CAUSES OF REDUCED INSULIN SENSITIVITY AND RESPONSIVENESS IN VITRO, REDUCED PERIPHERAL UPTAKE OF GLUCOSE AND GLUCOSE INTOLERANCE.

The striking difference in the behaviour of isolated adipocytes from both ob/ob fed ad libitum and ob/ob-RD mice (but not in lean mice) and fat pads from these mice suggests the presence of a mechanism outside the adipocyte which intervenes between the biologically active insulin molecule[48,51] and its receptor site on the fat cell; (it is now well demonstrated that the intact cell surface is required for the

biological response to insulin[172]). A rise in plasma free fatty acids may also be expected to reduce muscle responsiveness to insulin[222]; however, no increase in plasma free fatty acid concentrations have been observed in ob/ob mice[324].

Other authors have put forward the hypothesis that the early insulin resistance of the peripheral tissues (muscle and adipose tissue) leads to an accumulation of glucose in the blood after glucose loading from the fed state and this in turn stimulates the pancreas to produce increasing amounts of insulin This is indirectly supported by the work of Westman[33a] and others[46,48] who have shown that the pancreatic and serum levels of IRI reach their peak when the animals are about 6 months old and well after the hyperglycaemia has reached its zenith at 3 months of age. This has been taken as another example of the rodent pancreas' remarkable ability to respond to the functional demands made on it[64]. This proposal is not supported by the fact that the plasma IRI concentrations of ob/ob-RD mice,(who have normal blood glucose concentrations), although less than the ob/ob fed ad libitum values, are still nine times higher than the equivalent values found in lean mice (Table 16).

Hormonal resistance in ob/ob mice has also been observed with epinephrine by some investigators[57,58,199,202]. However, one report[277] which expressed lipolysis in adipose tissue per number of cells failed to find any impairment of response to epinephrine.

The results presented in this thesis suggest that insulin resistance in vitro is not an early lesion of the ob/ob mouse and is not a primary expression of the gene. It is, therefore, more likely to be an adaptation to hyperglycaemia

and hyperinsulinaemia caused by glucose intolerance. One of the hypotheses which has been advanced to explain the glucose intolerance has been the reduced peripheral uptake of glucose. The significantly reduced incorporation of glucose-U-^{14}C to glycogen in quarterdiaphragms from 2-4 month old ob/ob mice, and inactivity (p.161) (explicable as a result of hyperphagia) could lead to a hyperglycaemia, which would be followed by hyperinsulinaemia and tissue resistance to insulin. The complete unresponsiveness to insulin after 5-6 months of age maintains the hyperglycaemia (as muscle synthesis of glycogen in vitro is now normal) which in turn would stabilise the appetite at a new higher food adaptive intake, and, together with other mechanisms, stabilise the obesity. This theory has the advantage in fitting a time sequence to the various experimental facts available: (see p.161-162 however). A reduced glucose oxidation by adipose tissue has also been observed in fat pads incubated at concentrations of glucose in the medium not exceeding 20mM; however, in the presence of 80 mM glucose the oxidation was restored to normal[56]. As pads from ob/ob mice contain less adipocytes per unit wet weight, this suggests an increased uptake and metabolism of glucose in pads from ob/ob mice at high extracellular glucose concentrations. The normal glucose metabolism per unit wet weight of epididymal fat pads in very young ob/ob mice[56] is probably due to a comparable number of adipocytes per unit wet weight in pads from lean and ob/ob mice in this age group, as the adipocytes in the prelipogenic phase of obesity would not have had time to enlarge or would have just commenced to enlarge.

Other possible explanations which have been advanced for the hyperglycaemia of ob/ob mice have included a hyperglycaemic-glycogenolytic

factor which is antagonistic to insulin and which may act on the pancreas as it can be temporarily eliminated by diethyldithiocarbamate[328]. Relevant to this hypothesis, Gepts[47] described a three-fold increase in α cells and Clarke et al.[329] observed an increase in pancreatic glucagon after growth hormone administration in ob/ob mice. (see p. 210) It is also possible that ob/ob mice possess a defect in their livers which may not elaborate factors which augment glucose uptake in skeletal muscle[330].

4. THE SIGNIFICANCE OF THE HYPERINSULINAEMIA IN OB/OB MICE.

The sign most reliably associated with obesity in all pathologies, including the ob/ob and a "physiological" obesity such as the third trimester of pregnancy[71] is hyperinsulinaemia. Numerous factors and hormones are known to influence the insulin content and secretory capabilities of the pancreas[250-255,331-336], the most intruiging of which are anterior pituitary extracts and catecholamines in view of their suspected involvement in some of the signs of the ob/ob syndrome. Hepp[337] has postulated that a differential action of insulin on its possible the inhibition of lipolysis and its stimulation of lipogenesis could account for obesity in hyperinsulinaemic states. The inhibition of lipolysis in adipose tissue *in vitro*[337] and *in vivo*[85] is very much more sensitive to insulin than is its effect on lipogenesis from glucose.

Reduction of the overweight in human obesity reduces the hyperinsulinaemia that characterises that

condition[78-80]. Despite the indirect evidence for the secondary nature of the hyperinsulinaemia in ob/ob mice (v.s.), it remains a parameter, exaggerated by the administration of glucose, that persists in ob/ob-RD mice and in 5 week old ob/ob mice who were not diabetic, and has been shown to be a distinguishing characteristic of the adult fed or fasted ob/ob mouse's response to glucose. Lean mice showed no hyperinsulinaemia even when their blood glucose levels were comparably high. This would indicate a sensitisation of the pancreas in animals presented with a chronic excess of food. Thus, goldthioglucose-obese mice and hypothalamic obese rats and mice, who are hyperphagic during their period of rapid weight gain, are also hyperinsulinaemic[48,231,239,258-260], without any manifestation of an accompanying diabetes.[146] However, there is one report of hyperinsulinaemia in rats made obese by VMN-hypothalamic lesions where hyperphagia was not a cause of the obesity[240]. The hyperinsulinaemia which occurs in animals with normal blood glucose, as in hyperphagic goldthioglucose obese mice[237] and hypothalamic obese rats[48,231,239,258], may also be due to a delayed and prolonged response of plasma IRI to glucose administration, which would lead to hyperinsulinaemia in a hyperphagic animal showing repeated brief elevations of blood glucose. It is not known if the hyperinsulinaemia of hypothalamic obese animals disappears after their body weight has stabilised and their food intake returns to normal, (although it has been claimed that no such period of stable body weight occurs in goldthioglucose obese mice[338]).

It is possible that hyperinsulinaemia could be the cause of the tissue insulin resistance in ob/ob mice fed ad libitum. In fed ob/ob-RD mice, who are insulin-responsive

in vivo (on 2.5 g. food a day[54]), their continuing hyperinsulinaemia can be explained by the persisting reduction in insulin responsiveness of their epididymal fat pads (on 5 g. food a day). Perhaps severe undernutrition (2.5 g. food a day) is necessary for the complete return of insulin responsiveness and the normalisation of plasma IRI concentrations in ob/ob-RD mice. The close temporal association of in vitro peripheral tissue unresponsiveness to insulin and hyperinsulinaemia in many different pathologies makes it very difficult to distinguis which defect occurs first. This study of ob/ob mice at 2-4 months and 6-8 months of age has not succeeded in separating these two signs on the basis of age probably because the mice were not studied young enough when secondary adaptations had not yet developed. Although hyperinsulinism associated with maternal diabetes has been observed in neonates in the human [339-341,343] in the diabetic Chinese hamster[342] and in human foetuses[343], it is not known if it was accompanied by a peripheral tissue resistance to insulin in these cases.

Peripheral tissue resistance to insulin is a physiological adaptation that is reasonable to expect in an animal that is becoming rapidly obese through, probably, an increased food intake. Its increased development would finally help limit the obesity and might, by some unknown mechanism, stabilise the increasing food intake to a level commensurate with the new metabolic efficiency of the animal. The adjustment of the new food intake level would occur in a quantitative manner that is genetically determined, each species of obesity stabilising at an 'obese' body weight that is apparently 'ideal' for that particular strain or type of obesity. This is

presumably caused by variable hypothalamic sensitivity to glucose, (e.g. compare the different LD^{50} for goldthioglucose in different strains of mouse, p. 38), or some other substance associated with the fat content of the animal. The normal sensitivity and responsiveness to insulin of hyperphagic goldthioglucose-obese mice that has been reported in vitro[56,57] and in vivo[59,237] (but not by Katsuki[236]), concurrent with a moderate hyperinsulinaemia[48,258] may have been observed because these mice had been studied during their period of rapid weight gain. These are the characteristics of the lipogenic state that one would expect in ob/ob mice before they adapted to their developing obesity with a secondary adaptation of peripheral tissue resistance. It is not known if goldthioglucose-obese mice have been studied in the lipostatic period of their obesity when their food intake was not elevated. It is possible that a secondary peripheral tissue resistance to insulin will be detected in vitro in goldthioglucose-obese mice if studied when their body weight has stabilised.

5. POSSIBLE AETIOLOGY OF THE HYPERGLYCAEMIA IN THE OB/OB MOUSE.

As stated earlier, because of the complex metabolic changes introduced by the administration of a restricted diet to ob/ob mice, it would be naive to assume that the hyperglycaemia of ob/ob mice was purely due to their hyperphagia. There are many other causes of hyperglycaemia, most of which can be experimentally produced[344]. In particular, the permissive role of the adrenals[345] and the pituitary[346] are still not clearly understood. It has been suspected that the paradoxical reduction in glucose tolerance (after starvation)[344,349,350] which is markedly improved by feeding of carbohydrate prior to the glucose tolerance

test[347,350-352], is caused by a diabetogenic factor from the anterior pituitary[314] as this phenomenon disappears after hypophysectomy[353]. This phenomenon has also been shown to occur in the sand rat[354]. With this animal, Brodoff[354] has shown that median eminence lesions in the area of the arcuate nucleus have an ameliorative effect on the blood sugar concentrations of these animals. He has argued that interruption of the hypothalamic-hypophyseal reflex arc breaks the negative feedback from the hypothalamus, responding to hypoglycaemia or some related circulating substance, to the neurosecretory cells of the anterior pituitary.

When this is achieved in normal CBA mice by hypophysectomy, goldthioglucose fails to induce obesity[355]. However, the hypothalamus plays an important part, independent of the pituitary, in the development of obesity, as has been shown with hypophysectomised, VMN-lesioned rats which accumulate more body fat than hypophysectomised controls even when pair-fed[356]: this gain in weight has been largely attributed to the reduced oxygen consumption of these rats[357]. Similar studies on the ob/ob mouse have not yet been undertaken.

One other interesting hypothesis concerning the aetiology of the diabetes in these mice deserves mention.

Endoportal administration of insulin has been shown to effect the same degree of arterial hypoglycaemia as does peripheral administration of insulin[358]. It has been suggested, therefore on the basis of the responses of plasma glucose and insulin in man to high and low carbohydrate diets[359], that obesity can result

from the action of glucose and insulin on a "fed insulinized" liver (suggested by the raised glucokinase levels in ob/ob livers[320] - liver glucokinase concentrations increase with feeding in rats or when treated with insulin injections, and disappear in fasted or alloxan-diabetic rats[360]) and a "diabetic fasted" periphery possibly caused by the ineffectiveness or antagonism of insulin peripherally either by the excess secretion of, for example, growth hormone, ACTH or corticosteroids[361a], or by the splitting of endoportal insulin by the liver to release large amounts of insulin A-chains into the peripheral circulation[361b]. The latter event would lead to a reduced muscle glucose uptake[361c], but an unaltered stimulation of glucose metabolism in adipose tissue[361c]. A primary defect leading to hyperinsulinaemia would result in obesity by saturating the liver with glycogen and fat, leading to the increased passive release of triglycerides and β-lipoproteins from the liver to adipose tissue which would accumulate fat from triglycerides[362]. The greater number of heparin-secreting mast cells in ob/ob adipose tissue[31] would encourage this process. In time the peripheral tissues would become completely resistant to the hyperinsulinaemia, marking the end of the lipogenic phase. A hypertriglyceridemia has not been reported in ob/ob mice probably because the net release and uptake processes are efficient (viz. the turnover rate is high).

6. OTHER INVESTIGATIONS.

There have been five main lines of investigation other than those that have been described, which have contributed to the elucidation of the aetiology of obesity in ob/ob mice:

(i) Hyperlipogenesis from glucose and other carbohydrates.

(ii) Impaired lipolysis in adipose tissue.

(iii) Parabiotic experiments with ob/ob and lean mice.

(iv) Studies on the earliest detectable lesion of the ob/ob mouse.

(v) Studies on the sympathetic drive of ob/ob mice.

(i) Hyperlipogenesis.

Numerous investigations have been carried out to ascertain whether the grossly increased fat accumulation in ob/ob mice can be explained by either an increased lipogenesis from carbohydrate or fat in liver or adipose tissue, or an impaired fat mobilisation from adipose tissue, or a combination of the two mechanisms.

It was shown quite early in studies on the obese mice[31,121,285] that although the oxidation of ^{14}C-acetate was decreased, the *in vivo* incorporation of acetate-C^{14} into carcass and liver fatty acids was increased even after fasting both *in vivo* and *in vitro*. This was confirmed in more detailed *in vivo* experiments investigating the fate of labelled carbohydrates administered intraperitoneally or intragastrically when it was found that the hypertrophied liver from ob/ob mice incorporated 5-10 times more glucose and lactate into liver fatty acids[130, 286,363-365]. Incorporation into carcass lipids was 0.5 - 2.0 times greater in the ob/ob mice depending upon their age and dietary regimen[130,363,364]. *In vitro* experiments with the epididymal fat pad showed similar differences in that the incorporations of acetate, pyruvate or glucose into fatty acids and lipids per unit weight[31,121,366] or the esterification of ^{14}C-palmitate per fat cell[368] were increased only in

the absence of added glucose. Further, this increase was much greater in the young ob/ob mice in the lipogenic phase of their obesity than in older mice whose body weight had stabilised[130,285] and occurred apparently without an increase in energy requirements.

As Stauffacher[59] has concluded, enhanced lipogenesis as the primary disorder in ob/ob mice would not of itself explain the syndrome because it would probably result in an increased glucose tolerance. The latter would not, of course, occur if the hyperlipogenesis was due to an increased supply of FFA to ob/ob adipose tissue through more, or more active, lipoprotein lipase[362], especially stimulated by the heparin of the increased number of mast cells in ob/ob adipose tissue[31]. Lipogenesis in adipose tissue from this source would be independent of the source of C that would be needed (ref.130) and would be enhanced by an increase in dietary C intake.

(ii) Impaired lipolysis.

There are reports in the literature that there is a decreased capacity for FFA mobilization in the fat pads[57,58, 199-201] or adipocytes[202,203] from ob/ob mice. However, this is contrary to the findings of Abraham et al.[324] who have shown that the regulation of triglyceride breakdown in vivo as measured by the free fatty acid content of the plasma is normal in ob/ob mice. The normal rate of turnover of ^{14}C-palmitate in the plasma of ob/ob mice[369] suggests that changes in plasma FFA removal did not account for these results. This discrepancy between the in vivo and in vitro studies could be explained by differences in morphology of the fat pad described in the present work. Thus, as in the case of insulin, there may be insufficient access of catecholamines to the fat cells. Thus,

ACTH-induced lipolysis, impaired in pads from ob/ob mice, when expressed per unit wet weight of tissue[200], has been found to be completely normal in isolated adipocytes from ob/ob mice[244] when the results are expressed on the basis of DNA content, or in pads when expressed per unit number of cells[277]. The arguments against impaired lipolysis being a primary defect in ob/ob mice have been summarised in a recent paper from this laboratory[324]. Further, inhibition of FFA mobilisation in vivo with lipolytic blocking agents has no effect on the hyperglycaemia of diabetes or the blood sugar level of normal subjects over 60 minutes, except when there coexists an exercise demand[370]. Any impairment in the availability of FFA would partly influence the metabolic mixture used in oxidative metabolism[371] and would tend to increase the metabolic utilisation of glucose especially by muscle and adipose tissue and this is patently not the case.

(iii) Parabiosis.

Hervey[372] and Han[373] have both shown that when a hypothalamic-obese rat is parabiosed to a lean littermate, it increases its food intake and obesity at the expense of the lean parabiont. Bray[245] has had similar results after parabiosis of genetically obese rats to their lean littermates and similar findings are also reported by those who have parabiosed lean and ob/ob littermates together[374,317] and in the parabiosis of db/db mice with normal controls[375]. This failure to detect a circulating humoral agent important for the development of the obesity, may have been due to the parabiosis not having been performed early enough at a critical time in the

initiation of the obesity because there does exist one report, which has not been confirmed, of a decrease in the obesity of the ob/ob mouse after parabiosis to a lean littermate[376]. The same author, however, finds that transplanted adipose tissue always takes on the characteristics of the host tissue, suggesting that an intrinsic lesion of adipose tissue is not the cause of the obesity[377]. The most interesting observations have come from the experiments of Strautz[378,379] who, after transplanting ob/ob and lean pancreatic islets into the peritoneal cavities of ob/ob and lean mice, found that ob/ob mice lacked a factor, present in lean mice, that was small enough to diffuse through a millipore filter and which prevented the development of obesity. These results are extremely difficult to comment on, especially as our knowledge of the proportions of α1- and α-2 cell types in the pancreas of lean and ob/ob mice is as yet incomplete[380]. Strautz' factor, which may possibly turn out to be very important in the aetiology of obesity in the ob/ob mouse, may ,like so many of the endocrine abnormalities of this syndrome, be pituitary-linked and reversible under paired-feeding conditions. It is possible that this factor is produced by the α-1 cells of the pancreas which are known to have a potent inhibitory effect on insulin release by isolated islets[381]; this is particularly relevant in view of the persistence of hyperinsulinaemia in the ob/ob-RD mouse.

(iv) The earliest detectable lesion.

The search for the primary biochemical lesion, (assuming there is only one), in the obese-hyperglycaemic syndrome has been approached by studying the mice before their obesity or hyperglycaemia became apparent and by studying young established obese mice in the fasting state with normal blood glucose concentrations. The former approach suggested that a higher fat:fat-free dry weight ratio was an abnormality that became apparent before the overweight, hyperglycaemia and hyperinsulinaemia[33a,33b]. Recently, Danielsson et al.[382] have shown, by injecting glucose intraperitoneally into suckling mice at 23 days of age and measuring any glycosuria present after one hour, that glucose intolerance was present only in future obese mice (assuming equivalent renal thresholds for glucose). Westman[33a] found *in vivo* insulin resistance in *ob/ob* mice at 24 days of age. The very young age at which the *ob/ob* syndrome becomes apparent suggests that intrauterine or postpartum factors may play an important role in the genesis of the obesity. In the rat, for example, it is known that early nutritional experiences have a permanent effect on the number of adipose cells the animal is endowed with[384], possibly by causing intrauterine hyperinsulinaemia[383] and this presumably is a strong determinant of the final degree of obesity and metabolic alterations that will occur.

(v) The sympathetic drive of ob/ob mice.

The increased susceptibility to cold[38,39], the lowered blood supply to adipose tissue per unit weight and the *in vitro* unresponsiveness of adipose tissue to

catecholamines[57,58,199-203] are signs suggestive of an impaired sympathetic drive in ob/ob mice. It has been shown that adrenal demedullated rats that have had their ability to mobilise FFA from adipose tissue blocked with nicotinic acid mimic the behaviour of ob/ob mice, and rapidly lose temperature and die when placed in the cold after this treatment[370]. However, adrenalectomy does not prevent the development of the obesity in ob/ob mice[385]. As adrenaline is known to have an antagonistic effect on insulin in diaphragm muscle in rats[88], one would not expect the observed increased insulin resistance in their muscle *in vitro* if ob/ob mice had ineffectual or absent circulating catecholamines. However, reduced stimulation of adipose tissue sympathetic nerve endings possibly under direct hypothalamic control may be one mechanism for the fat accretion of obesity that is independent of the hypothesis of a pituitary humoral substance[386,387]. Further, the fact that immuno-sympathectomised rats are not nearly as obese as are ob/ob mice suggests that a pure deficiency in the peripheral sympathetic nervous system cannot be the only abnormality to account for the massive obesity seen in ob/ob mice. In support of this, it has been shown that guanethidine does not improve the glucose tolerance of the sand rat, but reserpine (1 mg./kg.) in a single dose was effective in improving it[388], possibly by depleting the sympathomimetic amines in the central nervous system. Clearly the interrelationships of the hypothalamus and the sympathetic nervous system are closely involved in this syndrome.

7. CONCLUSION.

In conclusion, after nearly twenty years of research, little is known of the locus at which the genetic defect acts in the ob/ob mouse. The main reason for this is undoubtedly the inability to obtain fully controlled experimental conditions in an animal so different from its control and in a field of study so easily complicated by the conditions under which the animals are investigated. Much useful information has been derived from the experiments where one variable has been controlled, as in the use of ob/ob-RD and hypothalamic-obese mice. It is difficult to focus on the lesion in the ob/ob syndrome when divergencies from the control lean mouse abound, in experiments which leave one in considerable doubt as to whether the data has resulted from an experimental variable, a method of expressing results or a gene defect. Many of the results described in the present work are common to obesities with differing aetiologies and prompt the hypothesi that they are physiological manifestations of the response to increased fat storage. The essential physiology in this animal is still shrouded in ignorance: the contributions to the blood glucose from intestine, liver and kidney have hardly been studied, and make futile any discussions on the elevated blood level of glucose and its relation to the reduced peripheral uptake of glucose.

The work reported here has shown that ob/ob mice possess peripheral tissues whose poor responsiveness to insulin was abolished or improved when they were maintained on a regimen of reduced food intake. Further, by studying isolated

adipocytes, it was shown that the insulin resistance of adipose tissue was mainly determined by factors outside the adipocyte. It was suggested that the reduced adipocyte density in epididymal adipose tissue from ob/ob mice is an important variable in the altered metabolism and response to hormones observed, and considerable understanding into the mechanism of the control of fat storage in adipose tissue would ensue after study, particularly in utero, of the factors which control and influence the number of adipocytes, and undifferentiated mesenchymal cells and the vascular supply of this tissue. The plasma insulin concentrations of ob/ob mice, in the fed or fasted state or after chronic restriction of food intake, were higher than in lean mice and did not correlate with the blood glucose concentration. The exaggerated response of the plasma IRI to ingested glucose in fasted ob/ob mice fed ad libitum was, however, considerably reduced in fasted ob/ob-RD mice or young ob/ob mice. This suggests that the response of the pancreas of ob/ob mice to ingested glucose has a different time course to that found in lean mice and that it is influenced by the blood glucose concentration, (presumably via β-cell glycogen content), and/or the concentration and rate of absorption of glucose from the alimentary tract.

Undoubtedly there is considerable evidence which points to the involvement of the pituitary gland in this syndrome. We still do now know how it is involved, what part metabolic homoeostasis plays in the development of the gland in utero, and whether it is always concerned in the various patterns of endocrine response which stabilise the obesity in

different genetic obesities, (nearly always manifested by hyperphagia). Advances must await experimental techniques that will monitor the effects of the hypothalamic control on the autonomic nervous system, on the alimentary system and on the target endocrine organs of the pituitary gland, and that will facilitate the understanding of the complex neural circuits that underlie one of the most important and primitive homoeostatic mechanisms: the control of fuel supply and storage.

A P P E N D I X

ANALYSIS OF THOMSON RAT AND MOUSE RESEARCH DIET SUPPLIED BY PILSBURY'S LTD. BIRMINGHAM, ENGLAND.

INGREDIENTS

Barley	Wheat by-products
Maize	Dried yeast products
Wheat	Mineral supplement
Oats	Vitamin supplement
English meat meal	English white fish meal

Dried milk powder

Metabolisable Energy 3185 Kcals/kg. Carbohydrate 51%

CALCULATED ANALYSIS

	%		p.p.m.
Crude Protein	21.48	Fe	40
Crude Oil	3.05	Mn	18
Crude Fibre	4.7	Cu	6
Digestible Crude Protein	18.51	I	0.816
Digestible Oil	2.4	Co	0.00736
Digestible Fibre	1.94	Zn	4.8
Arginine	1.11		
Lysine	1.19		1.U./lb
Methionine	0.395	Vitamin A	2096
Cystine	0.3	Vitamin D_3	262
Tryptophane	0.224		
Histidine	0.468		mgm/lb
Leucine	1.56	Vitamin E	7.082
Tyrosine	0.535	Thiamine	2.69
Isoleucine	0.965	Riboflavine	2.83
Phenylalanine	0.888	Niacin	22.5
Threonine	0.785	Pantothenic Acid	8.13
Valine	1.17	Choline	602
Glycine	1.31	Biotin	0.29
Ca	1.54	Folic Acid	0.26
P	1.10	Pyridoxine	2.4
Ca:P	1:0.7	Inositol	Not less than 10
Na	0.339		
Cl	0.05		mcgm/lb
Mg	0.202	Vitamin B12	5.4

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