INVESTIGATIONS ON OBESITY

AND

EXPERIMENTAL HYPERLIPAEMIA

by

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SUMMARY

The results of experiments in an animal model for obesity, namely obese hyperglycaemic mice (ob/ob) are described and their relevance to the study of obesity and endogenous hyperlipaemia in man is discussed.

Alternating periods of fasting and refeeding reduced the body weight, blood glucose, and plasma insulin levels of obese hyperglycaemic (ob/ob) mice, which also showed an increase in physical activity. A return to normal feeding habit reversed these changes. ob/ob mice showed no weight loss when treated with various doses of tri-iodothyronine (T_3), 'slim' ob/ob mice did not breed, and an unexpected number of lipomas at the tail of the pancreas was discovered in these animals.

A study of changes in the distribution and numbers of gut hormone-producing cells in these mice was commenced and the possibility that these 'insulin-releasing' gastro-intestinal hormones may be involved in the development of obesity is discussed.

ACKNOWLEDGEMENTS

Throughout this work I have been grateful for the valued guidance, interest, patience and encouragement of Professor V. Marks to whom I express a very sincere gratitude. My thanks are also due to many other members of staff and post-graduates of the Department for their co-operation and rewarding discussions, especially Dr. J. Fry, Miss C. Jones, and Dr. J.W. Bridges for their collaboration in cultured hepatocyte experiments; Dr. R.S. Jones for his valued guidance and help in the liver perfusion studies. My appreciation also to Mr. Andrew Taylor and Mr. Peter Wood for their help with the preparation of this Thesis.

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TO: Hana, my dear wife, for her love, devotion and patience.

NON-STANDARD ABBREVIATIONS

bw	Body weight
Ci	Curie
EDTA	Ethylene diamine tetracetic acid
EGTA	Ethylene glycol-bis-(β -aminoethylether) N,N'-tetra acetic acid
FFA	Free fatty acids
GIP	Gastric inhibitory polypeptide
GLI	Glucagon-like immunoreactivity
HE	Haematoxylin and eosin
IP	Intraperitoneal
IRG	Immuno reactive glucagon
IRI	Immuno reactive insulin
IRP	Intestinal insulin-releasing polypeptide
IU	International units
IV	Intravenous
MEM	Minimum essential medium
MLD	Minimum lethal dose
PAS	Periodic acid schiff
PBS'A'	Phosphate buffered saline lacking calcium and magnesium
PCA	Perchloric acid
RIA	Radioimmunoassay
SC	Subcutaneous
SEM	Standard error of the mean
T_3	Triiodothyronine
TPB	Tryptose phosphate broth
U	Unit
VIP	Vasoactive intestinal polypeptide

Other abbreviations are those recommended by the 'Biochemical Journal' chemical formulae are those in common use.

C O N T E N T S

C H A P T E R 1

GENERAL INTRODUCTION

Obesity which may be defined as an excess of body fat, is the most important nutritional disease in the affluent countries of the world (Kannel, LeBauer, Dawber and McNamara 1967; Baird 1973; Mann 1975). A survey in the United States showed that about two thirds of the population is overweight to the extent associated with diminished life expectancy (Office of Health Economics 1969).

In the Framingham Heart Disease and Epidemiology Study, obesity was the factor most closely associated with the frequency of atherosclerotic cardiovascular disease and was a determinant of the risk factors for hypertension, hyperlipaemia and diabetes (Kannel et al 1967; Gordon and Kannel 1973; Pfeiffer and Laube 1974; Clark and Duncan 1972; Walker 1972).

The association of obesity with many disabling but non-fatal diseases is also well documented; for example, osteo-arthritis of the knee is common in middle-aged obese women (Leach, Baumgard and Broom 1973).

There is known to be a genetic component in the causation of obesity, but it has been difficult to separate this genetic effect from the contribution made by environmental factors. Genetic factors appear to have a greater influence on the extent of fat deposition in the trunk than in the limbs (Brook, Huntley and Slack 1975).

Weight reduction of moderately obese men, on a weight-reducing diet, resulted in a marked fall in their plasma cholesterol and triglyceride levels (Leelarthaepin, Woodhill, Palmer and Blacket 1974). It was therefore suggested that both body weight and the composition of the diet are important determinants of plasma lipid levels. Weight gain after maturity makes an important contribution to Type II and IV hyperlipaemia.

Weight reduction improves life expectancy, is often the most useful step in treating osteoarthritis of the knee and may prevent other disorders associated with obesity (Dublin 1953).

Many methods to reduce the weight of obese subjects have been tried with various degrees of success (Garrow 1974). Substantial weight reduction is often very difficult to achieve - indeed some authorities despair of the efficacy of many forms of dietary advice for making people lose weight permanently (Stunkard 1972; Garrow 1974; Lancet Editorial 1974). Anorectic drugs are of limited usefulness in the treatment of obesity. Drug treatment never seems to achieve weight-loss greater than 20 kg-average-(Duncan and Munro 1968; Steel, Munro and Duncan 1973; Blundell 1974).

In the face of these disheartening results, more and more drastic treatments for gross obesity have been tried. Despite the unpleasant side-effects, Jejunal and Ileal - bypass surgery are gaining ground as a line of treatment in severe obesity (Baddeley 1973; Bliss 1973; Gazet, Pilkington, Kalucy, Crisp and Day 1974; Clinical Nutrition Editorial 1975).

Since treatment is so difficult, attention has increasingly been directed towards prevention, although we still lack satisfactory methods for the prevention of obesity (Garrow 1974).

In the last 25 years, there has been extensive research into obesity and hyperlipaemia, both in man and in experimental animals. Despite all the efforts, however, the mechanisms which are ultimately responsible for obesity and hyperlipaemia remain unknown (Albrink 1974; Bray and York 1971; Bruch, 1974; Galton 1971; Garrow 1974; Gordon 1970; Howard 1975; Jequier 1975; Mahler 1974; Rabinowitz 1970; Schachter and Rodin 1974; Scheig 1974; Vague 1969; Vague and Boyer 1974; Welsh 1962; Whelan and Silverstone 1974).

The role of various hormones in producing and/or maintaining the obese state and hyperlipaemia has not been resolved. Insulin and glucagon, two hormones, which influence carbohydrate and lipid metabolism, have been implicated in the pathogenesis of obesity and hyperlipaemia (Marks and Samols 1968; Marks 1973; Schade and Eaton 1974).

Attempts to find correlations between hyperinsulinaemia, hypertriglyceridaemia (frequently associated with obesity) and obesity have not yielded consistant results (Bernstein, Grant and Kipnis 1975; Leelarthaepin et al 1974; Tremolieres, Sautier, Carre, Flament and Plumas 1974). Hyperinsulinaemia was thought by some workers to be due to a primary β-cell abnormality.

A mechanism has been recently postulated for both genetic and acquired endogenous hyperlipaemias seen in man and in experimental animals (Eaton and Schade 1973; Eaton, Schade and Conway 1974). It is suggested that the hypolipaemic action of glucagon, which is less marked in various hyperlipaemic states (for example, dietary, cobalt inducing, or genetic hyperlipaemia) might be responsible for the development and/or the maintenance of endogenous hyperlipaemia.

The evidence for the possible role of some of the gut hormones in the regulation of insulin secretion, reviewed recently by Grossman and his colleagues (1975), has opened up a new era in the search for the primary defect responsible for obesity and associated disorders such as hyperlipaemia and diabetes.

Ethical and practical difficulties are encountered with experiments on human subjects. Therefore, there is a great need for experimental animal models with features resembling those found in human obesity, hyperglycaemia and hyperlipaemia, for use in the study of these states and influencing factors.

Obese hyperglycaemic mice (ob/ob) and rats with cobalt chloride induced hyperlipaemia, have been used as experimental models in the present study.

C H A P T E R 2

MATERIALS AND METHODS

Chapter 2: Contents

A. Materials

I - Animals

Obese Hyperglycaemic Mice (ob/ob)

Mice, homozygous for the obese gene and bred from a mixed strain into which the ob gene had been incorporated (Abraham, Dade, Elliott, and Hems, 1971) were used together with their lean littermates (Fig.2:1). Animals were aged 4-6 months at the beginning of the studies described in the following Chapters and were bred in the University of Surrey Animal Unit.

II - Chemicals

Routine chemicals - Analar grade - were obtained from BDH, Poole; other chemicals, radiochemicals and media were supplied by various companies which are listed in Appendix I.

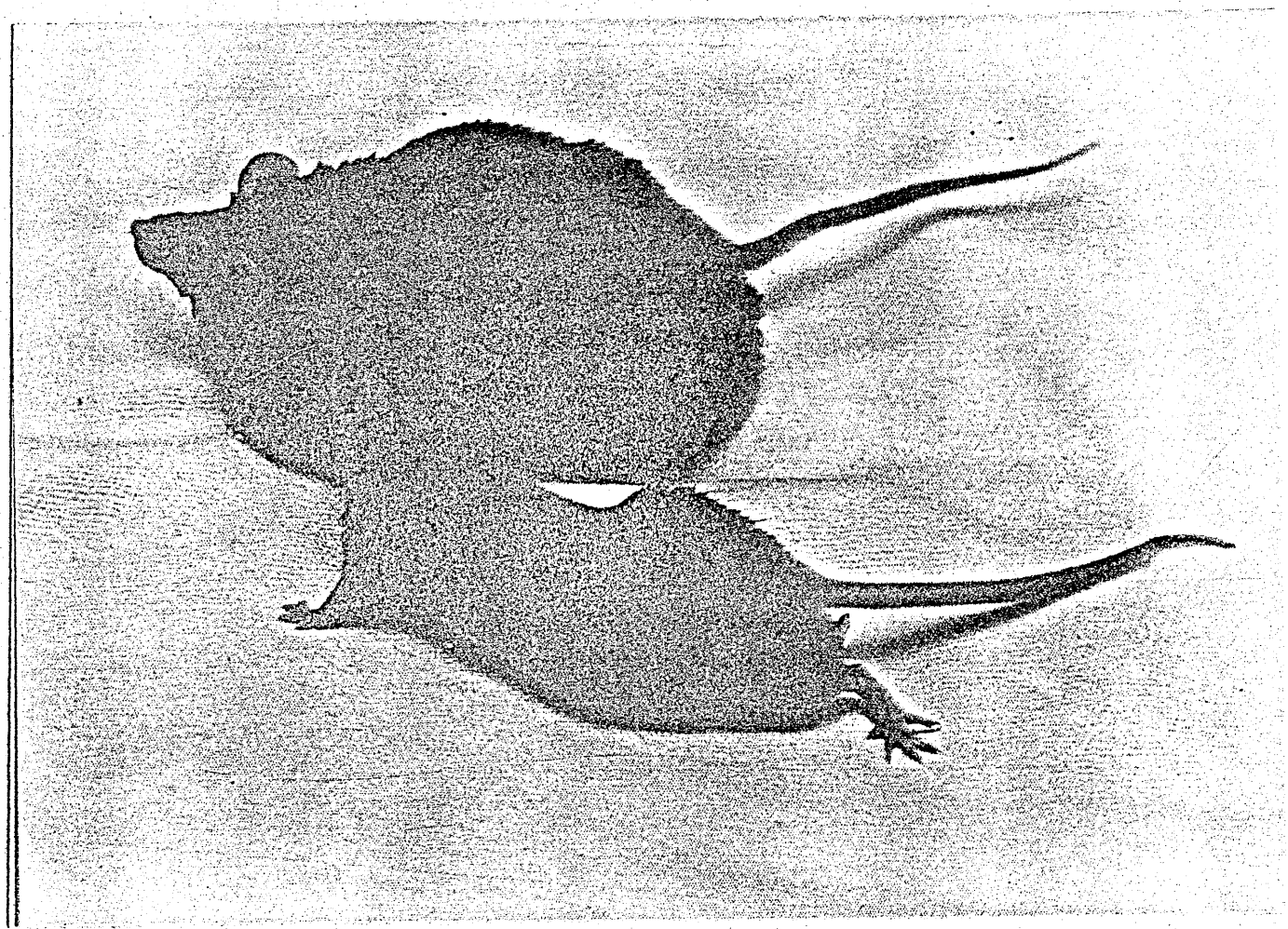


Fig. 2:1

An obese hyperglycaemic mouse and its lean littermate aged 6 months.

B. Methods

1. Measurement of Radioactivity by Liquid Scintillation Countin

All radioactivity measurements were made using a Packard Tri Carb Liquid Scintillation Counter, Model 3320. The scintillation mixture contained PPO (0.4%) and POPOP (0.1%) in toluene/Triton X-100; 2:1 (vol/vol).

For counting, 20 µl of medium perfusate or diluted bile was pipetted into scintillation vials containing 6 ml scintillant. The vials were shaken and counted for 10 minutes.

The counting efficiency in case of ^{14}C was about 80% and for ^{3}H about 42%.

2. The Measurement of Glucose and Insulin Levels

Samples for glucose measurements including blood were collected into 0.05 ml capillary pipettes and discharged into 0.95 ml 2% perchloric acid (PCA). The tubes were then centrifuged and kept at 4°C until measurement.

Glucose was measured in the Auto-analyser (Technicon Ltd.) using an automated GOD-perid method (Catalogue no. 15703, supplied by Boehringer Manheim Ltd.). This method uses the chromagen ABTS (ammonium salt of 2,2' azino-di (3-ethyl-benzo-thiazoline-6-sulphonic acid)) as redox indicator, with glucose oxidase and peroxidase (Kahle et al 1970).

Samples for insulin assay, including plasma, were collected into heparinised micro-centrifuge tubes (Gelman-Hawksely) and kept on ice until centrifuged. After centrifugation at 2,000 r.p.m. for 15 minutes the tubes were stored upright at -20°C until analysis. The portion of the tube which contained plasma was cut off just prior to determination.

Insulin levels were measured by a double antibody radioimmunoassay procedure, described in detail by Shabaan (M.Sc. Thesis 1973).

3. Measurement of Plasma Lipids

Fresh, unhaemolysed, serum was used to measure some of the plasma lipid constituents, i.e. triglyceride, cholesterol and free fatty acids (FFA).

Serum triglyceride levels were determined colorimetrically according to the method described by Fletcher (1968). This method was based on the principle that the glycerol released by saponification of the serum glycerides is oxidized to formaldehyde which reacts with acetylacetone to form a yellow dihydrolutidine derivative absorbing at 405 nm.

Cholesterol was measured colorimetrically following addition of acetic anhydride and concentrated sulphuric acid. The procedure and the reagents were supplied by Boehringer Manheim, London (Cat.No. 15969).

Serum FFA levels were also measured colorimetrically using a commercial kit (Boehringer Manheim (Cat.No. 15989)).

Quality control sera, Liponome^{R}, (BDH, Poole) and Seronorm^{R} (Nyegard-Norway), were used with all batches.

Other analytical techniques and methods which include autoradiography, atomic absorption spectroscopy and histological techniques, were also used. These techniques are described in appropriate sections in this Thesis.

C H A P T E R 3

INVESTIGATIONS ON OBESITY

Chapter 3: Contents

3.1: Introduction

The recessively inherited obese-hyperglycaemic syndrome in mice was first described in 1950 by Ingalls, Dickie and Snell. All mice homozygous for the gene (ob/ob) manifest the syndrome of which the most distinguishing feature is an excessive and abnormal deposition of fat (Mayer, Dickie, Bates and Vitale 1951).

In addition to the increased body weight these mice are characterised by insulin resistant hyperglycaemia (Mayer, Russell, Bates and Dickie 1953; Mayer 1953; Shull and Mayer 1956), together with high levels of circulating insulin (Stauffacher, Lambert, Vecchio and Renold 1967) altered fat metabolism (Hellman, Larsson and Westman 1962; Mayer and Jones 1953) and greatly enlarged functionally hyperactive islets of Langerhans, of which about 90% are β-cells (Gepts, Christophe andMayer 1960; Hellman, Brolin and Hellerstrom 1961; Hellman and Peterson 1960).

Other endocrine abnormalities are recorded in the ob/ob mice; Naeser (1974) showed that ob/ob had elevated plasma corticosteroids compared with their lean controls. Edwardson and Hugh (1975) showed that they have adrenal hypertrophy, increased corticosteroid secretion and considerably increased levels of pituitary ACTH.

Cycling periods in female obese mice (ob/ob) is either irregular or infrequent, however, the ova of ob/ob mice are fertile (Coleman and Hummel 1968).

Male obese mice (ob/ob) have considerably reduced volume and nuclear size of Leydig cells (Hellman, Jacobsen and Taljedal 1963; Lidell and Hellman 1966). A return of fertility to ob/ob mice of restricted body weight was reported by Lane and Dickie (1954).

Conflicting evidence about the role of thyroid in the development of obese-hyperglycaemic syndrome in ob/ob mice were reported.

No abnormalities either in thyroid weight or histology, have been detected in ob/ob mice (Bray and York 1971). The thyroid status of the ob/ob mouse, as measured by the uptake of ^{131}I, was shown to be normal (Goldberg and Mayer 1952; Wykes, Christian and Andrews 1958). However, this was recently challenged by Joosten and Van Der Kroon (1974) who suggested that ob/ob moucse is hypothyroid.

Thyroid function in the obese human subjects, as measured by radio-iodine thyroid uptake showed some differences from normal weight controls. The dynamic thyroid tests seem to indicate that the 'lazy thyroid' is due to a functional impairment in the control of the hypothalamus and/or pituitary (Riviere, Roger, Nogue, Emperaire and Latapie 1975).

Bray, Melvin and Chopra (1973) reported small decrease in the body weight of six grossly obese subjects, as a result of treatment with triiodothyronine (T_3).

Bray and York (1971) suggested that a hypothalamic defect might be involved in expression of the genetic abnormalities in ob/ob mice. Belloff-Chain, Edwardson and Hawthorn (1975), show that the insulin-releasing effect is not produced by ACTH, GH or prolactin in ob/ob mice, but possibly by a pituitary insulin-releasing factor which might be corticotrophin-like intermediate lobe peptide (CLIP) - (Edwardson 1975 - personal communication). This polypeptide was isolated and identified in the pig by A. Scott and his colleagues (Scott, Lowry, Ratcliffe, Rees and Landon 1974; Scott, Lowry, Bennett, McMartin, Ratcliffe 1974).

Roos, Martin, Westman-Naeser and Hellerstrom (1974), measured immunoreactive growth hormones levels in ob/ob mice and lean controls. They concluded that the insulin resistance of obese-hyperglycaemic animals was unlikely to be caused by pituitary hypersecretion of growth hormone.

Obese mouse (ob/ob) was also found to be characterised by a decrement in the concentration of insulin receptors per cell. Insulin receptors in liver, fat and thymic lymphocytes

are similarly affected, and in the lymphocytes, a decrease in receptor concentration is associated with a loss in insulin-stimulated amino iso-butyric acid transport. Total fasting for 24 hours or restriction of food intake to normal levels for a period of weeks, elevated insulin binding to normal or near normal levels (Freychet, Laudat, Laudat, Rossein Kahn, Gorden and Roth 1972; Kahn, Neville, Gorden, Freychet and Roth 1972; Kahn, Neville and Roth 1973; Soll, Goldfine, Roth and Kahn 1974).

It is now well recognised that intestinal hormones are concerned in the regulation of glucose homeostasis (McIntyre, Holdsworth and Turner 1965; Marks and Samols 1970) but their absolute identity and relative importance are uncertain (Pearse 1974; Grossman et al 1975).

Two recently isolated polypeptides, intestinal insulin releasing polypeptide (IRP) (Turner and Marks 1972; Turner, Shabaan, Etheridge and Marks 1973; Shabaan 1973; Shabaan, Turner and Marks 1974 a, b) and gastric inhibitory polypeptide (GIP) (Brown, Mutt and Pederson 1970; Brown, Pederson, Jorpes and Mutt 1969) were shown to produce dramatic effects on serum insulin levels in response to intravenous glucose in the rat (Dupré, Ross, Watson and Brown 1973; Rabinovitch and Dupré 1974; Turner, Etheridge, Marks, Brown and Mutt 1974) and in baboon (Turner, Etheridge, Jones, Marks, Meldrum, Bloom and Brown 1974).

Since the hormones of the gastrointestinal tract may play a part in the regulation of blood glucose levels through the release of pancreatic hormones, estimation of number and distribution of gut hormone-producing cells in ob/ob mice (Polak, Pearse, Grimelius and Marks 1975) was the first direct evidence of a possible role of gut hormones in the development of obese hyperglycaemic syndrome in ob/ob mice. They showed a degree of hyperplasia of most of the endocrine (APUD) cells (apudosis) in all regions of the gastrointestinal tract in ob/ob mice. In addition they also showed a significant difference in both number and hormone content of GIP, and

enteroglucagon (gut glucagon cross-reacting material, glucagon-like immunoreactivity, GLI) cells ($P < 0.001$) in obese mice as compared to lean mice. Differences in the case of secretin, gastrin, vasoactive intestinal polypeptide (VIP) cells were not great in the obese mice, but in the obese mice both gastrin and secretin cells were present in larger numbers in the lower intestine, whereas in the lean mice they were predominant in the upper intestine.

Despite all the information available, however, the mechanism ultimately responsible for the obese hyperglycaemic syndrome remains unknown.

Many of the current methods, used for body weight reduction in obese human subjects, are not satisfactory (Mann 1975). This is because they either fail to produce marked weight reduction in many patients (as in the low carbohydrate diets) or they are too hard to be tolerated by the majority of obese subjects (as in the complete food intake restriction for long periods).

A possible new approach to the problem of body weight reduction was tested in obese hyperglycaemic mice (ob/ob) using alternate periods of fasting and refeeding.

The following studies were carried out:

1. Measurement of body weight reduction in ob/ob mice using alternate periods of fasting and refeeding.

2. The effect of body weight reduction on blood glucose, plasma insulin, physical activity and possible breeding from 'slim' ob/ob mice.

3. The effect of triiodothyronine on body weight of ob/ob mice.

4. The effect of body weight and plasma insulin reduction on numbers and distribution of gut hormone-producing cells in ob/ob mice; in order to establish whether the obesity is due to hyperinsulinaemia or another primary abnormality causing both manifestation.

3.2 The Reduction of Body Weight in Obese Hyperglycaemic Mice (ob/ob)

(1) Reduction of body weight, plasma insulin and blood glucose levels, by alternate fasting and refeeding regimes in obese hyperglycaemic mice (ob/ob)

Obese hyperglycaemic mice (ob/ob) aged 5-6 months at the beginning of the studies, had been fed on normal rat chow diet and water ad libitum.

Blood for glucose and insulin measurement was obtained either from the tail vein or by heart puncture at the conclusion of the experiments.

Physical activity was measured simultaneously in an experimental animal and its control using two activity wheels (tread wheels) connected by microswitches to a single channel recorder (Servoscribe-Venture). Rotation of either of the two activity wheels was received as a pulse by the recorder and registered as a deviation from the midline on the same side of the recorder as the activated wheel (Fig.3.2:1). The microswitches on the activity wheels operated regardless of the direction of rotation.

The activities of the test mouse and its control were compared for 60 minutes and expressed as oscillation per hour.

(a) 72 hours fasting, 24 hours 'ad libitum' feeding regime

Two groups of six obese mice of the same age group (5-6 months) were weighed at the beginning of the study (Fig.3.2:2). Food was withheld from the test group for 72 hours followed by a 24-hour period during which access to food was allowed ad libitum. This cycle of fasting and refeeding was repeated during seven weeks (Fig.3.2:3-4). Water was freely available at all times. At the end of seven weeks normal feeding was resumed. After four weeks the animals were sacrificed. The control group was maintained on normal feeding throughout the eleven-week period.

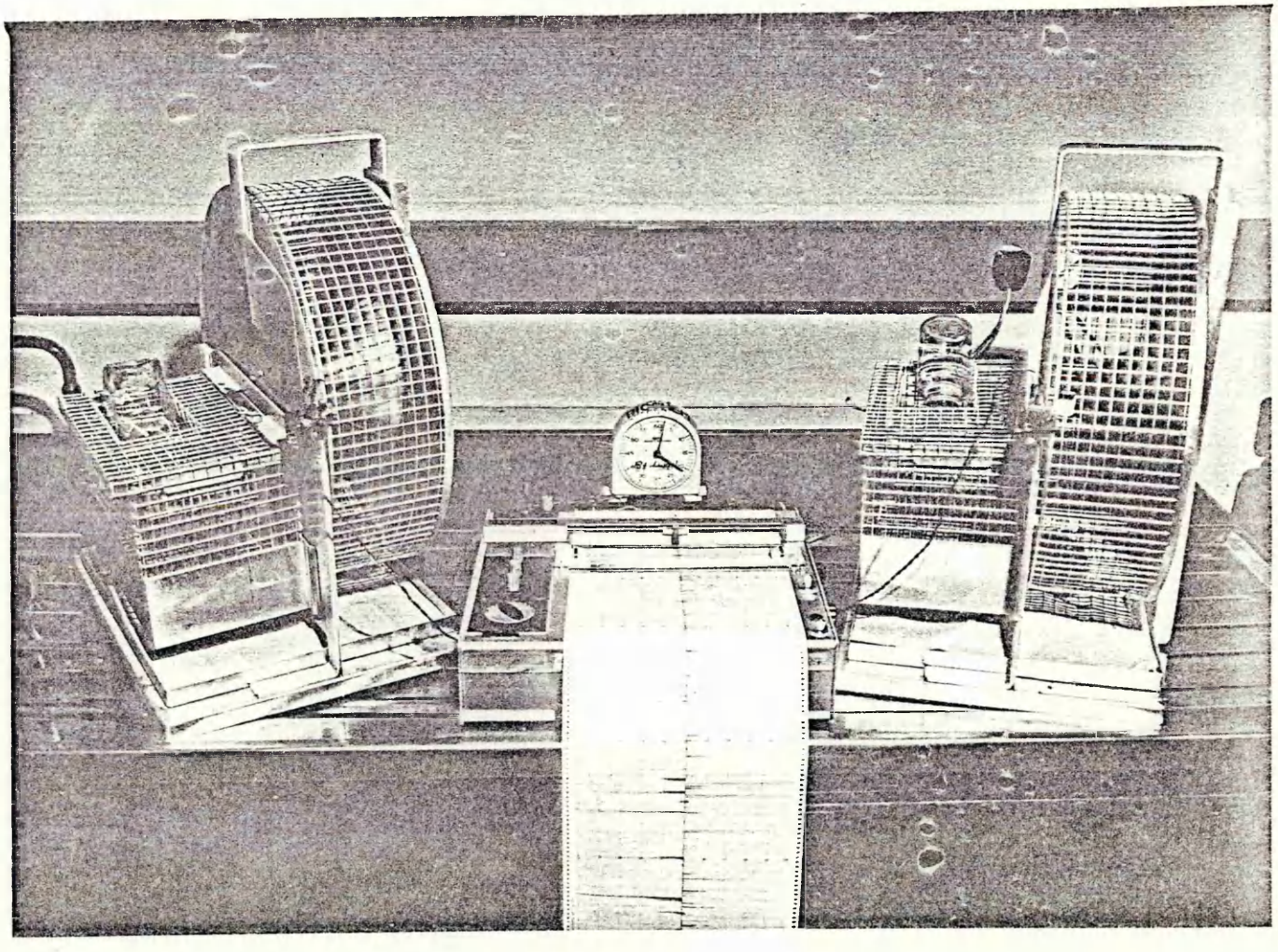


Fig. 3.2:1 Apparatus used to measure the physical activity.

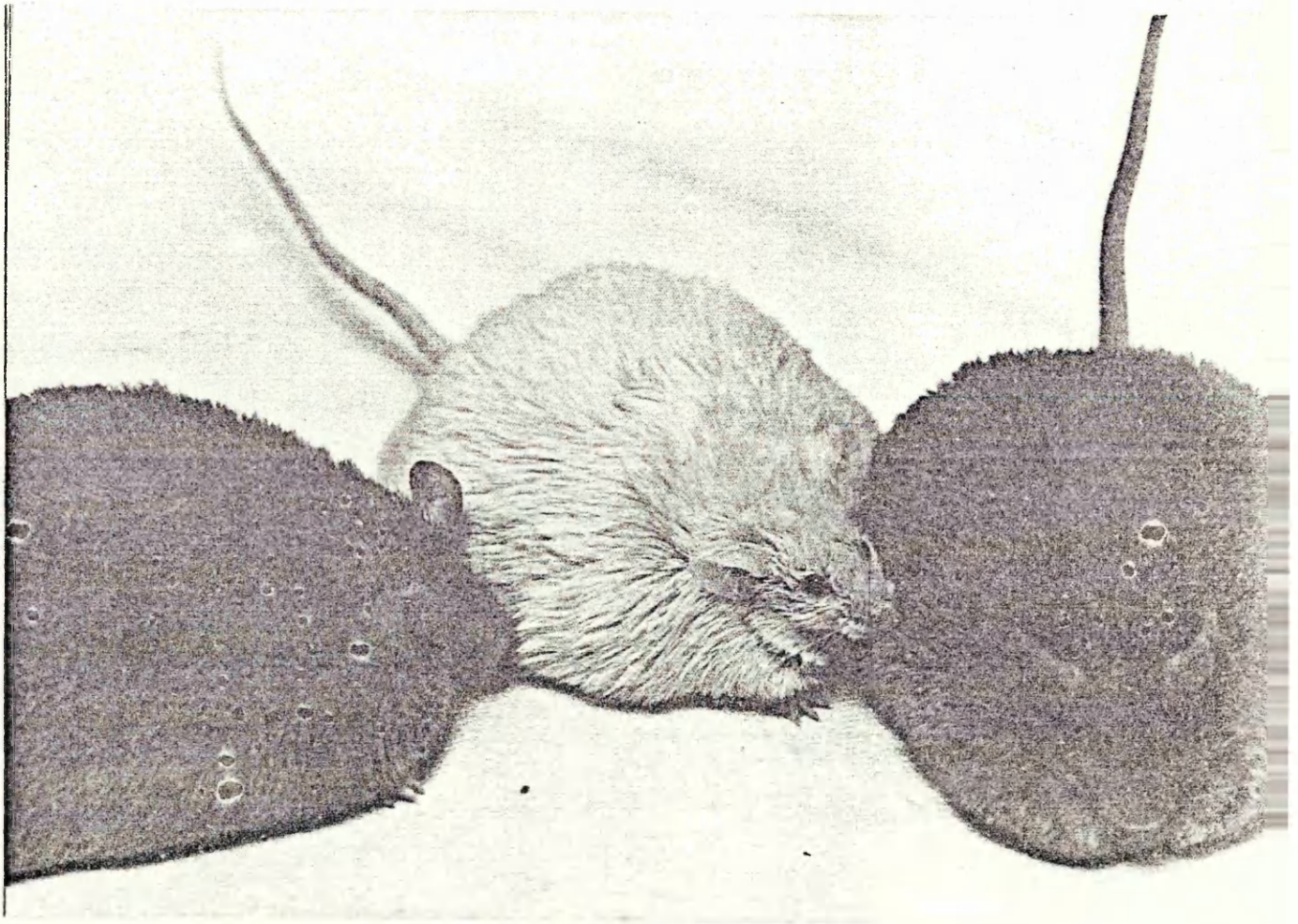


Fig. 3.2:2 Three out of the six test obese hyperglycaemic mice at the beginning of a weight reduction regime (72 hours fasting, 24 hours ad libitum feeding).

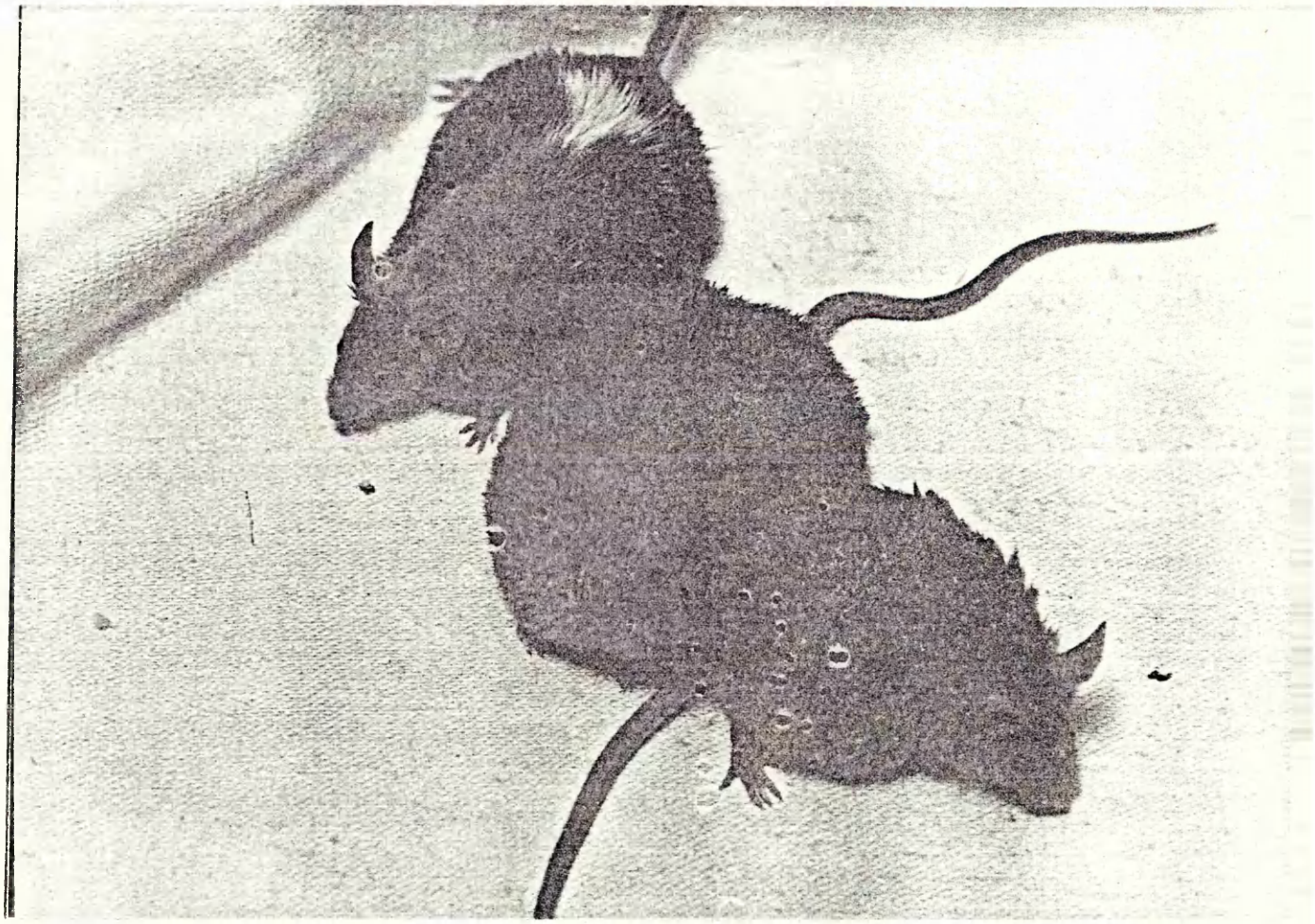


Fig. 3.2:3 Three out of the six test obese hyperglycaemic mice after seven weeks of a 72 hours fasting, 24 hours ad libitum feeding regime.

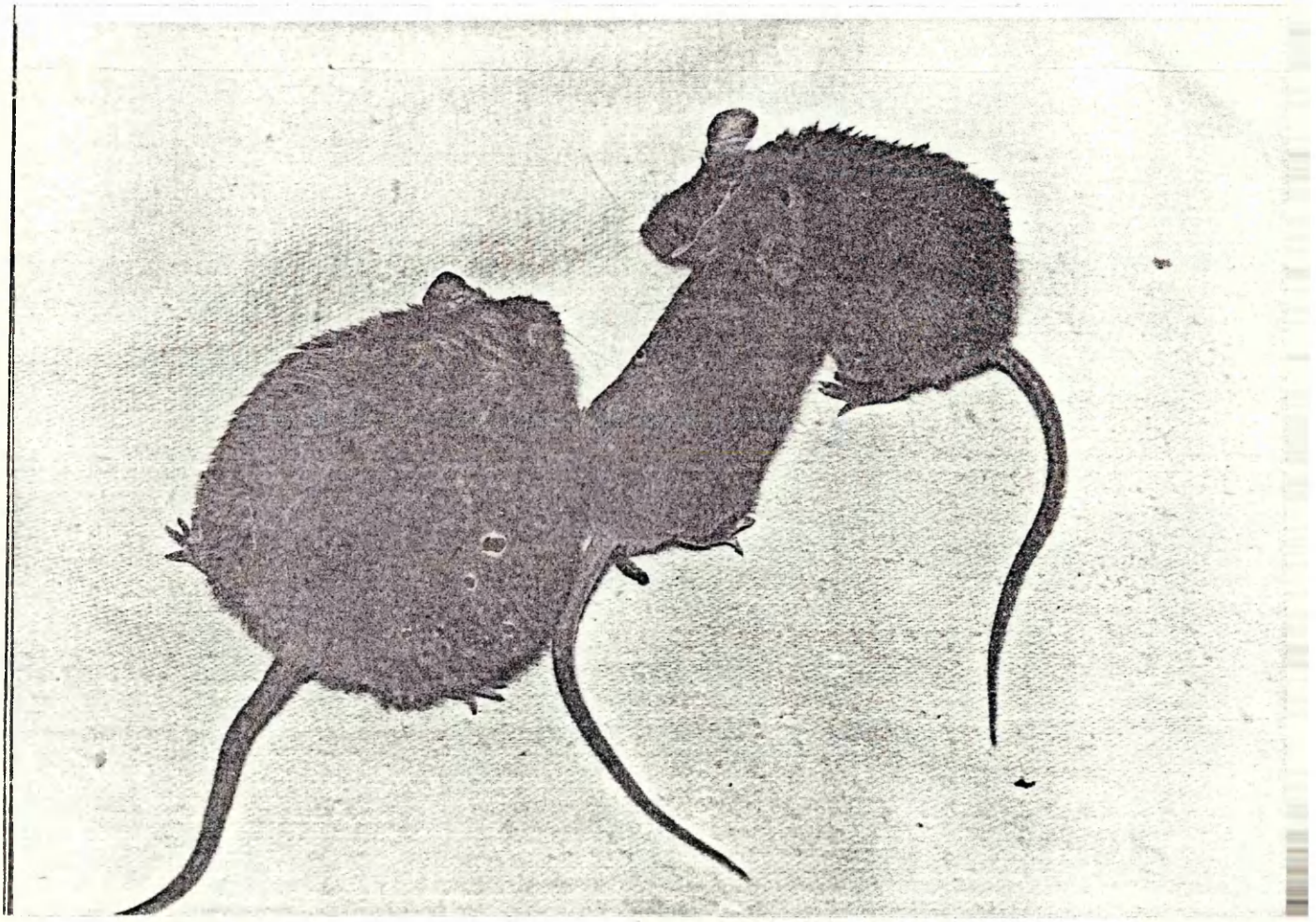


Fig. 3.2:4 An obese mouse (right) after seven weeks on a 72 hours fasting, 24 hours ad libitum feeding regime, its lean littermate and its obese control, fed ad libitum throughout the seven weeks experimental period, are shown for comparison.

Body weight was recorded weekly for both groups; in the test group measurements were made at the end of the feeding day. The results are shown in Fig. 3.2:5.

Weight loss was more or less constant throughout the period of fasting and refeeding, and averaged 6% per week, totalling 41% at the end of seven weeks. On resumption of normal feeding during the 8th-11th week the rate of weight gain averaged 9% per week.

A similar experiment was performed using twenty younger obese mice, aged four months (they did not reach their maximum weight).

The average weights (g) during this experiment are shown in Table 3.2:I.

Weeks	0	7	11
Obese controls (10)	95	97	97
Obese test (10)	91	47	94

Table 3.2:I

During this experiment two of the obese controls died towards the end of the experiment, whereas none of the test mice died during the period of the experiment.

(b) 48 hours fasting, 24 hours 'ad libitum' feeding regime

In this experiment the animals (five mice in each group) were treated in the same manner as above except that the fasting period was reduced to 48 hours (Fig. 3.2:6). Physical

Weight reduction in ob-ob mice on 72 hours fasting/24 hours ad libitum feeding regime.

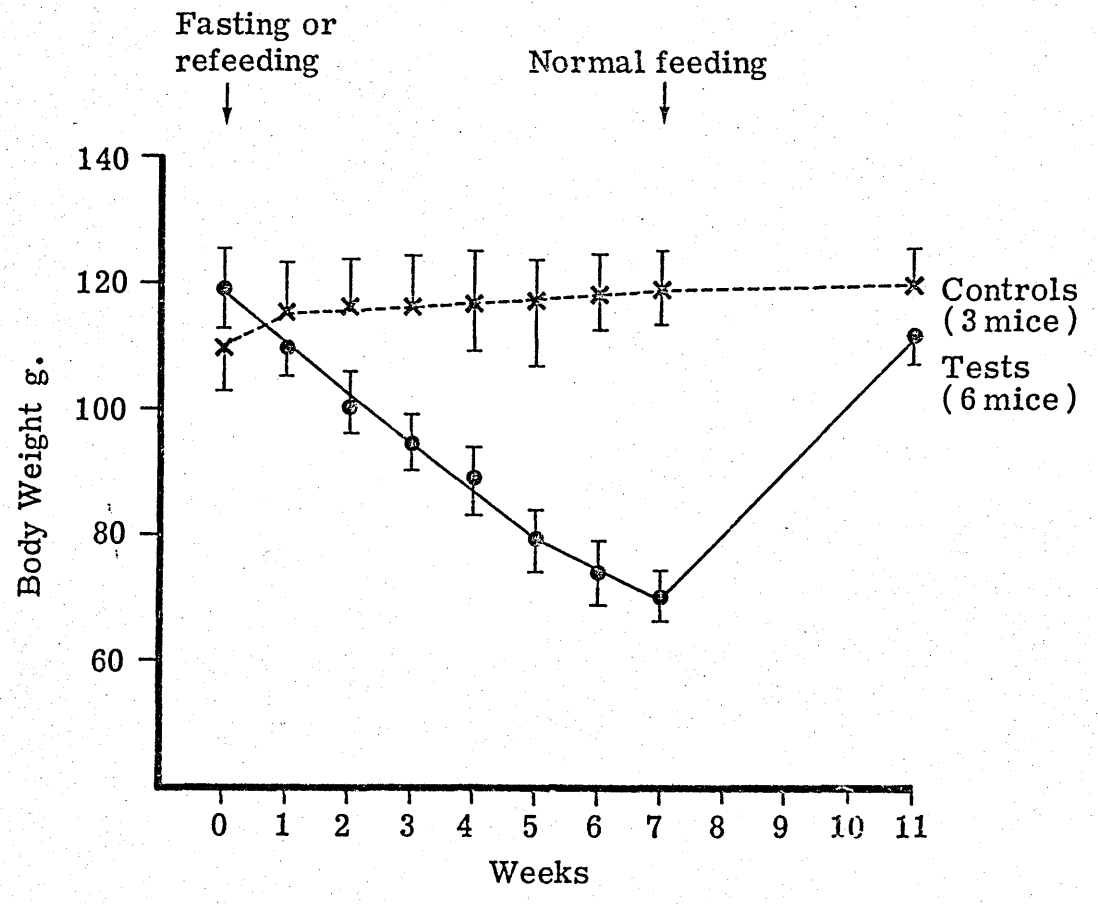


Fig. 3.2:5 Body weight (M± SEM) of test animals during seven weeks of 72 hours fasting/24 hours ad libitum feeding regime, followed by four weeks normal feeding. Normal weight of the control group also shown.

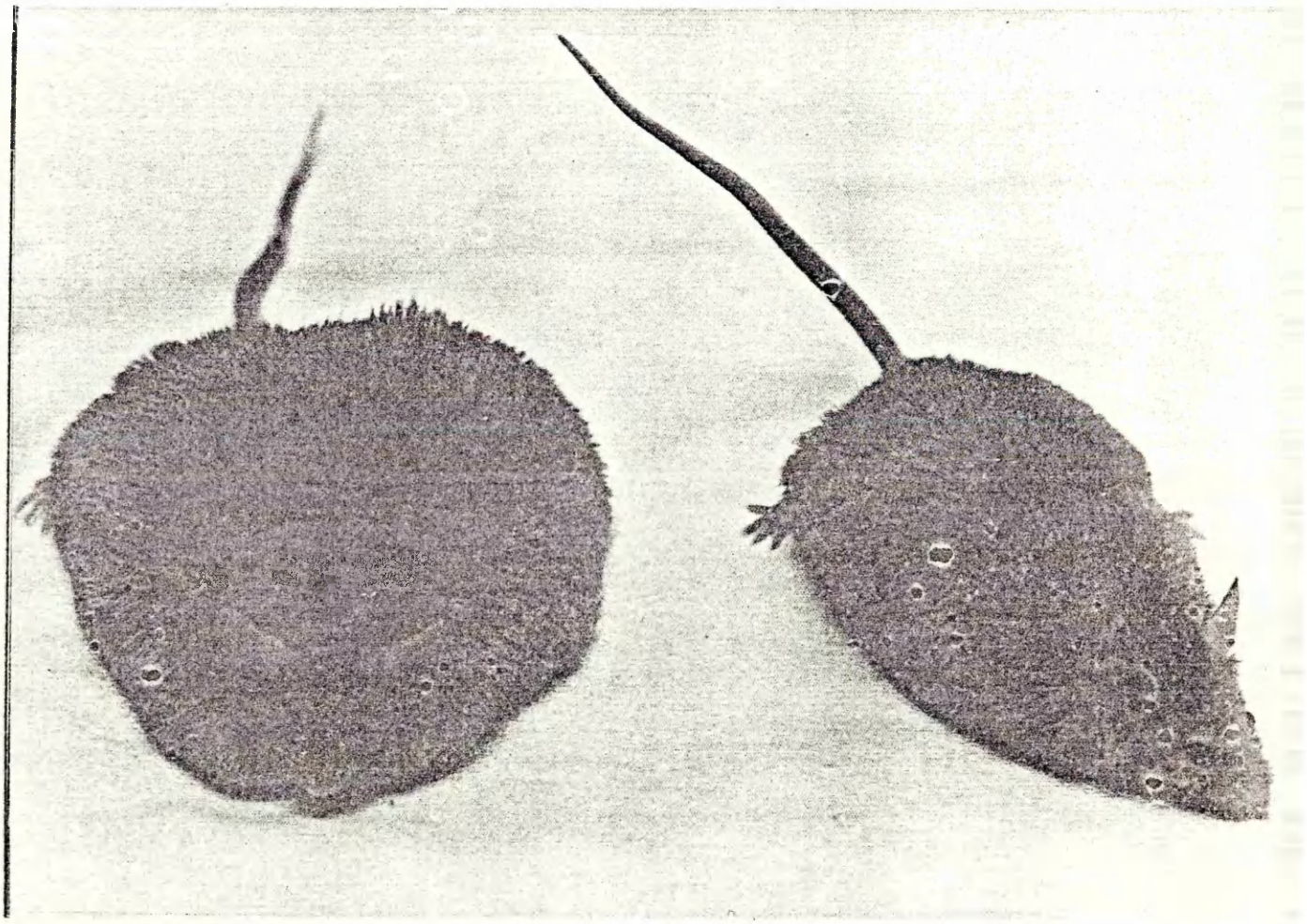


Fig. 3.2:6 Control obese mouse fed ad libitum (left), and its obese littermate after seven weeks of 48 hours fasting, 24 hours feeding regime (right).

activity, body weight, overnight fasting, blood glucose and plasma insulin levels were measured at various times throughout the experiment and the results are shown in Fig. 3.2:7. Changes in body weight correlated well with changes in blood glucose (correlation coefficient $r = 0.992$; $p < 0.001$) and changes in plasma insulin levels (correlation coefficient $r = 0.993$; $p < 0.001$). Physical activity in the test animals increased as their body weight decreased but had fallen again to basal values by the end of the experiment by which time body weight had returned to its initial level.

(2) The effect of triiodothyronine (T_3) treatment on body weight

Five obese mice and two lean littermates (aged 5-6 months) received a daily oral dose of T_3, dissolved in the drinking water. The dose was calculated from a knowledge of the animals average water intake and was initially given at a dose of 0.5 µg/kg body weight for three weeks and finally to 2 µg/kg for the rest of the eleven-week period. A control group of four obese mice did not receive any T_3.

The results are shown in Fig. 3.2:8 and indicate that despite the relatively high dose of T_3 used in these experiments there was no significant weight change in either the obese or lean treated mice.

(3) Breeding from obese mice (ob/lb) after weight reduction

Six young obese hyperglycaemic mice (ob/ob) - (three males and three females), aged ten weeks and weighed 66 g (mean), at the beginning of this study. The animal then underwent an initial period of four weeks of weight reduction, by a 48 hours fasting, 24 hour ad libitum feeding regime.

At the end of this period each 'slim' obese mouse (mean weight 37 g) was joined in a separate cage, with an opposite sex lean control mouse, of proven fertility (average weight 32 g). The pairs of mice were kept on normal feeding ad libitum for weight weeks, followed by another period (eight weeks) of alternate fasting and feeding as before.

Changes in physical activity, weight, glucose, and insulin in ob-ob mice on 48 hours fasting/24 hours ad libitum feeding regime.

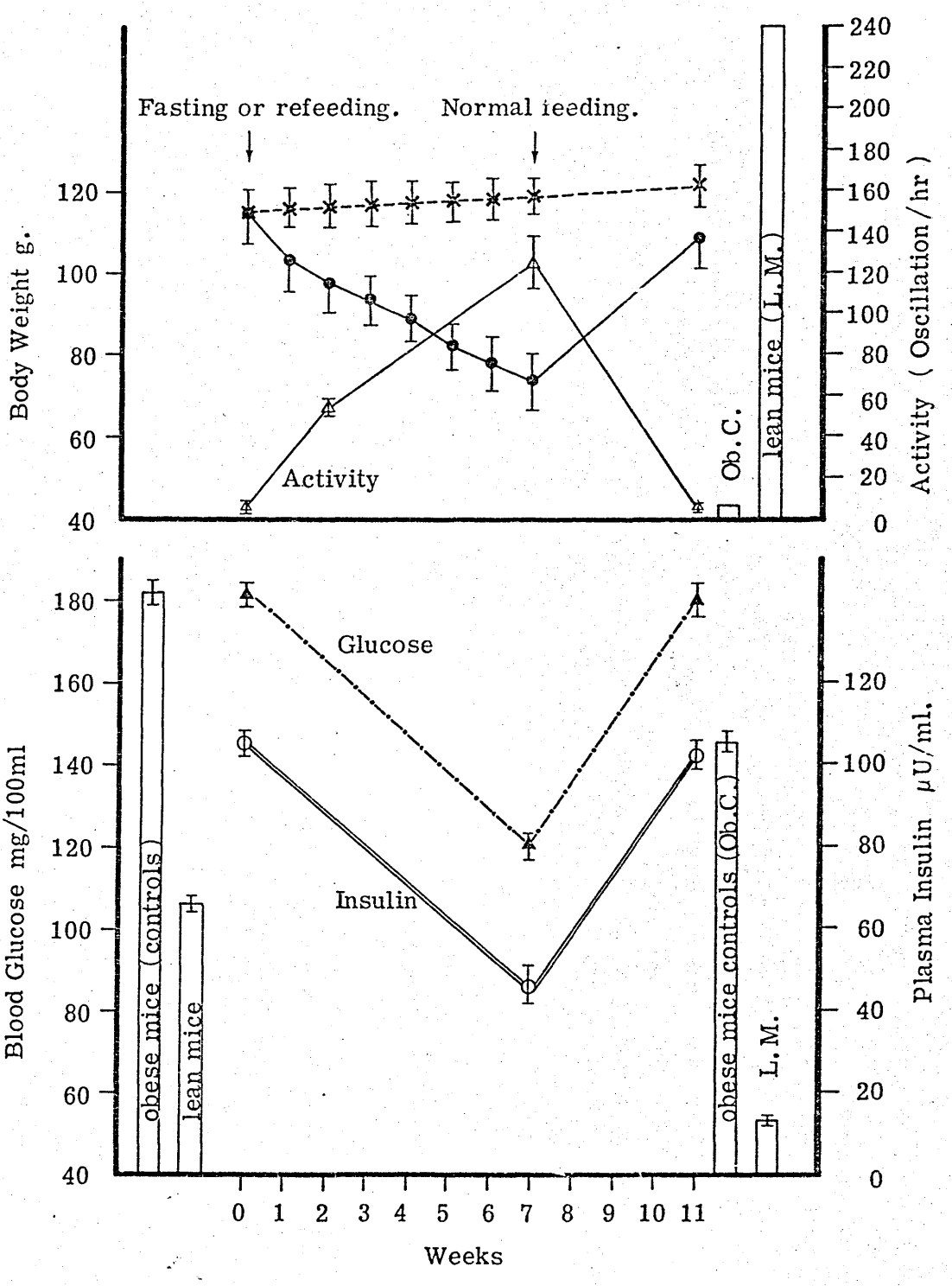


Fig. 3.2:7 Changes in body weight (●—● Test., ×---× control) physical activity (△——△) blood glucose (▲---▲) and plasma insulin (○═══○) levels in obese mice during seven weeks of 48 hours fasting/24 hours ad libitum feeding regime, followed by 4 weeks normal feeding.
Histograms show activity, blood glucose and plasma insulin levels in obese controls and lean lettermates.

Weight of ob-ob mice and lean littermates during treatment with oral tri-iodothyronine.

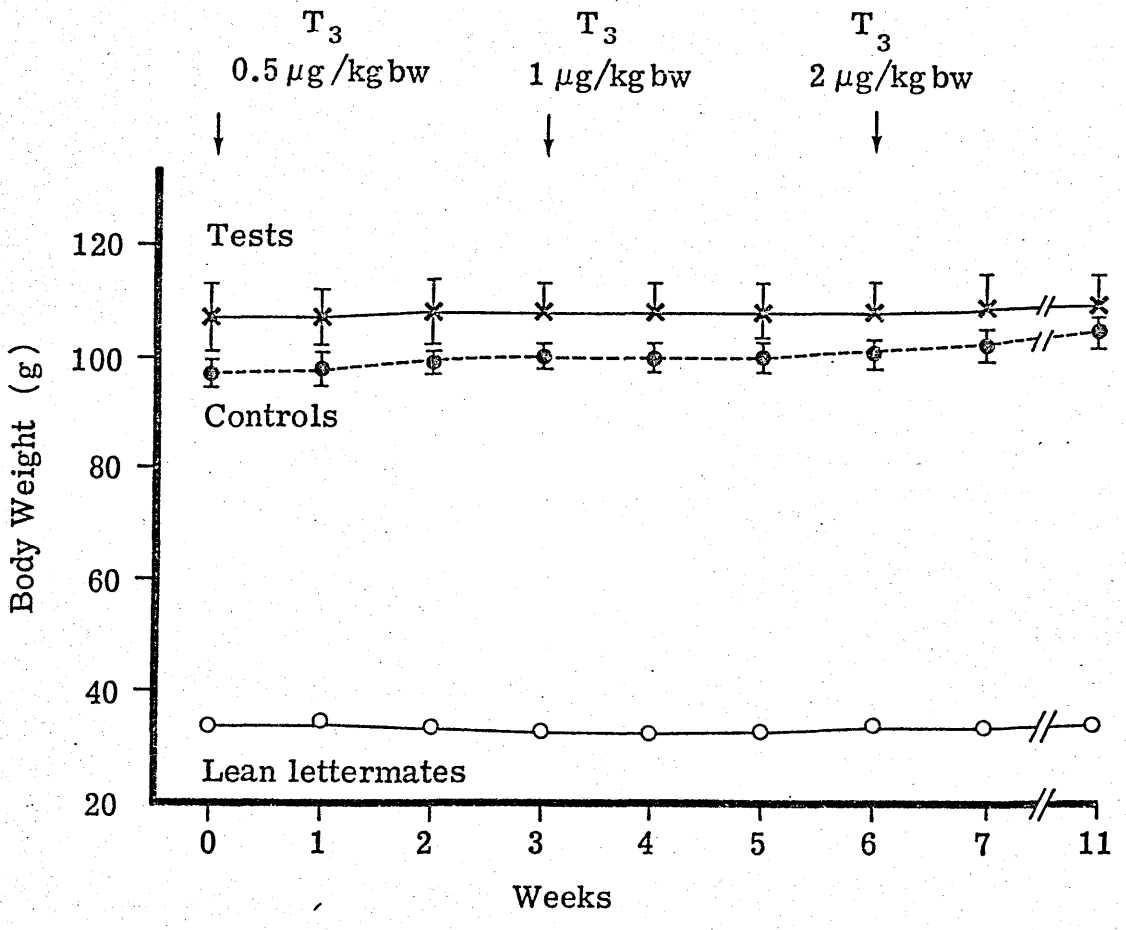


Fig. 3.2:8 Effect of 3 doses of tri-iodothyronine (T_3) upon body weight (Mean ± SEM).
×—× obese mice (10), o—o lean littermates (2), ●—● obese mice not receiving T_3 (10).

During the whole experimental period, no mating was observed between any pair, and none of the animals (lean or ex-obese females) became pregnant despite the fact that the weight of 'slim' obese mice was similar to that of the lean control mice.

DISCUSSION

The present study clearly shows that both the alternate fasting and refeeding regimes used are capable of producing marked reduction in body weight in obese mice (ob/ob) within a relatively short period (seven weeks).

The data suggests that weight loss was due mainly to a decrease in food intake rather than to the increase in physical activity. Since all the measurements of physical activity were carried out on the feeding day it is unlikely that food-seeking was an important factor in the increased activity observed.

Blood glucose and plasma insulin levels followed the pattern of weight change and the direct and close correlation between the various parameters was striking. By contrast, physical activity was inversely correlated with body weight, plasma insulin and blood glucose levels.

The rapid increase in overnight fasting blood glucose and plasma insulin during normal feeding (8-11 weeks) suggests that both are secondary manifestations of a prime cause (hyperphagia), which is responsible for the obese hyperglycaemic syndrome, rather than primary abnormalities. This point will be further discussed in Section 3.5.

Treatment with varying doses of triiodothyronine (T_3) failed to cause significant weight loss in obese mice (ob/ob) which had free access to food. It was therefore decided not to measure TSH, T_3, T_4 levels in the blood of these mice, as was originally planned.

The suggestion by Joosten and Van Der Kroom (1974) that the obesity of the ob/ob mice is due to hypothyroidism received no support from the present work which does not, however, preclude the possible involvement of other hormonal factor.

Breeding from obese mice (females and males) which underwent weight reduction regime, was not possible. This suggests that the infertility in ob/ob mice is not due to obesity as such, but to another primary abnormality.

3.3 Lipomas In Obese Hyperglycaemic Mice (ob/ob)

1 - Lipomas in Obese Mice Following Weight Reduction:

Whilst dissecting obese and lean control mice in preparation for histological and immunocytochemical experiments (see Section 3.4), a striking number of masses (lipomas) were found in some of the 'slim' obese mice which have been on a 72 hours (three days) fasting, 24 hours (one day) ad libitum feeding regime, for seven weeks (Fig.3.3:1).

Six out of twelve of the test mice had lipomas, all attached to the tail of the pancreas (Fig. 3.3:2-7). No lipomas were found in the twelve obese controls, nor the twelve lean control mice from the same experiment and dissected at the same time.

The lipomas varied in size from 8-20 mm in diameter (Fig. 3.3:2-7), some with necrosis (Fig. 3.3:2 and Fig. 3.3:6) and some without apparent necrosis (Fig. 3.3:4-5 and Fig. 3.3:7). On cutting, hard white masses were revealed as seen in Fig. 3.3:8.

The degree of necrosis and inflammation varied widely in degree, from one lipoma to another (Fig. 3.3:11-17). Also, fat cell infiltration into the pancreas varied in degree and in one lipoma cholesterol clefts were seen (Fig. 3.3:17).

A small piece of each lipoma and pancreas was cut and immediately frozen by Arcton (Freon -22) at -158^{o}C for immunocytochemical experiment. The remains were dissected and fixed in 10% formol saline for histological examination. A block from each lipoma and pancreas was later processed in Histokinette, and embedded in paraffin wax. Sections were cut and stained by haematoxylin and eosin.

The histology of the head and the middle part of the pancreas from both control and test obese mice was normal and showed the characteristic enlarged islets of Langerhans (Fig. 3.3:9-10), of which about 90% islet cells appeared to be

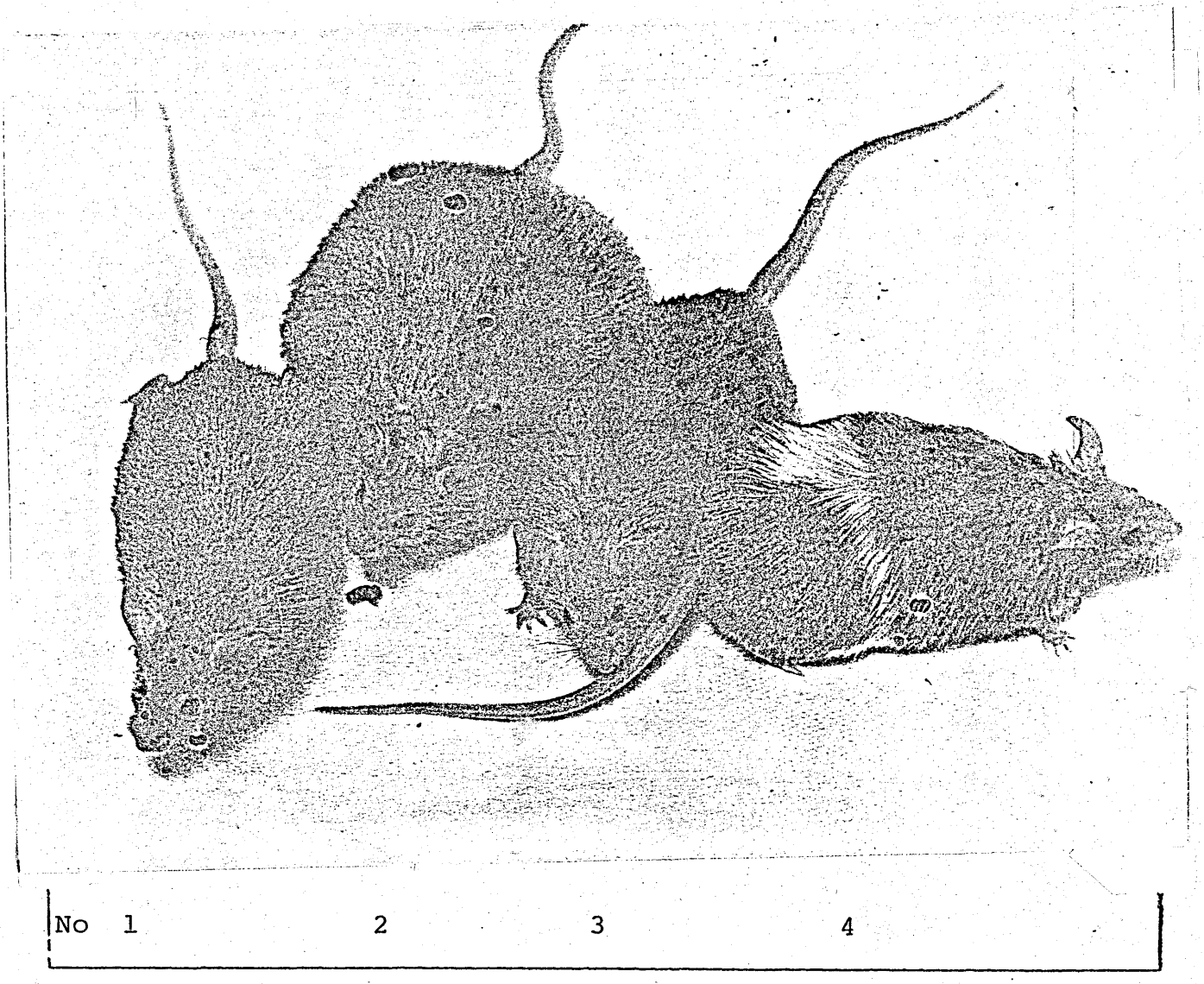


Fig. 3.3:1 Obese mice after weight reduction. Two test obese mice after seven weeks of 72 hours fasting, 24 hours ad libitum feeding (Nos. 1, 4) one obese control (No. 2) and a lean control (No.3) are shown for comparison.

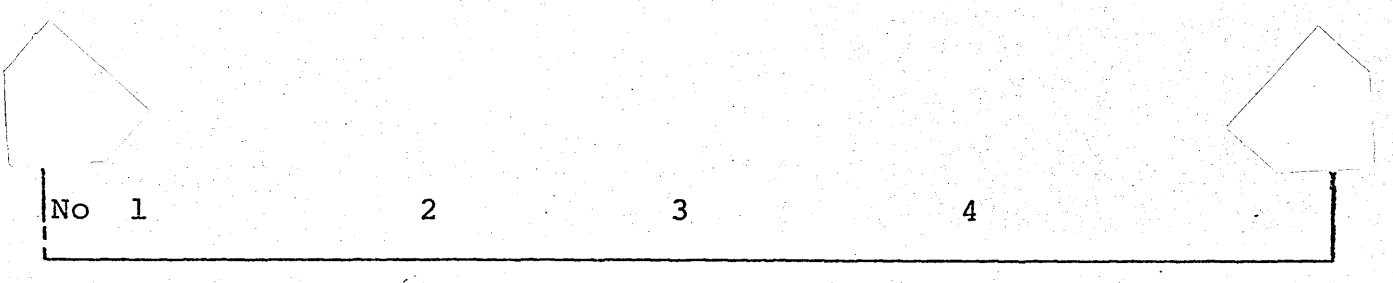


Fig. 3.3:1 Obese mice after weight reduction. Two test obese mice after seven weeks of 72 hours fasting, 24 hours ad libitum feeding (Nos. 1, 4) one obese control (No. 2) and a lean control (No.3) are shown for comparison.

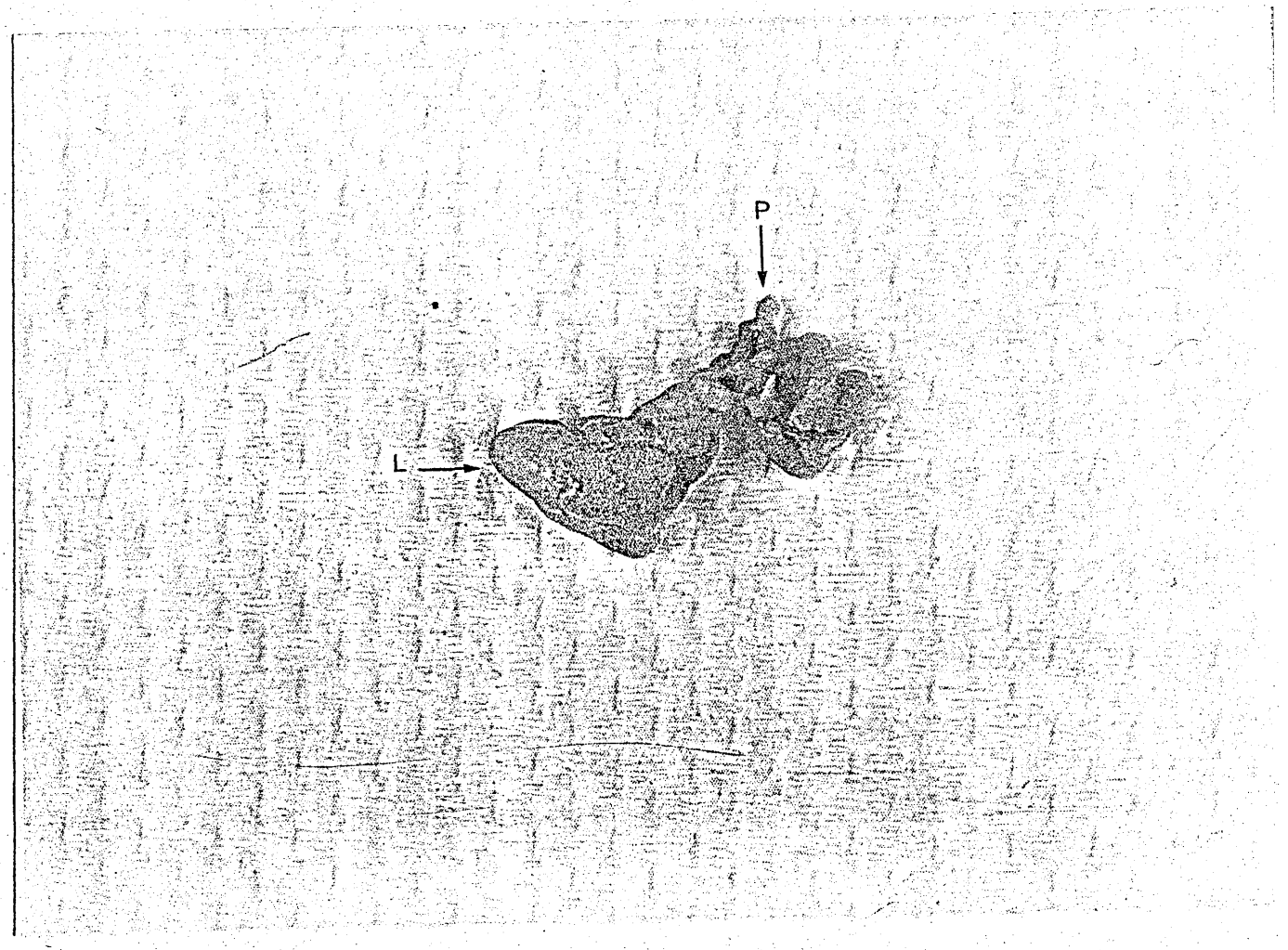


Fig 3.3:2 A lipoma attached to pancreas from eight months old obese mouse, after seven weeks of three days fasting, one day feeding regime.

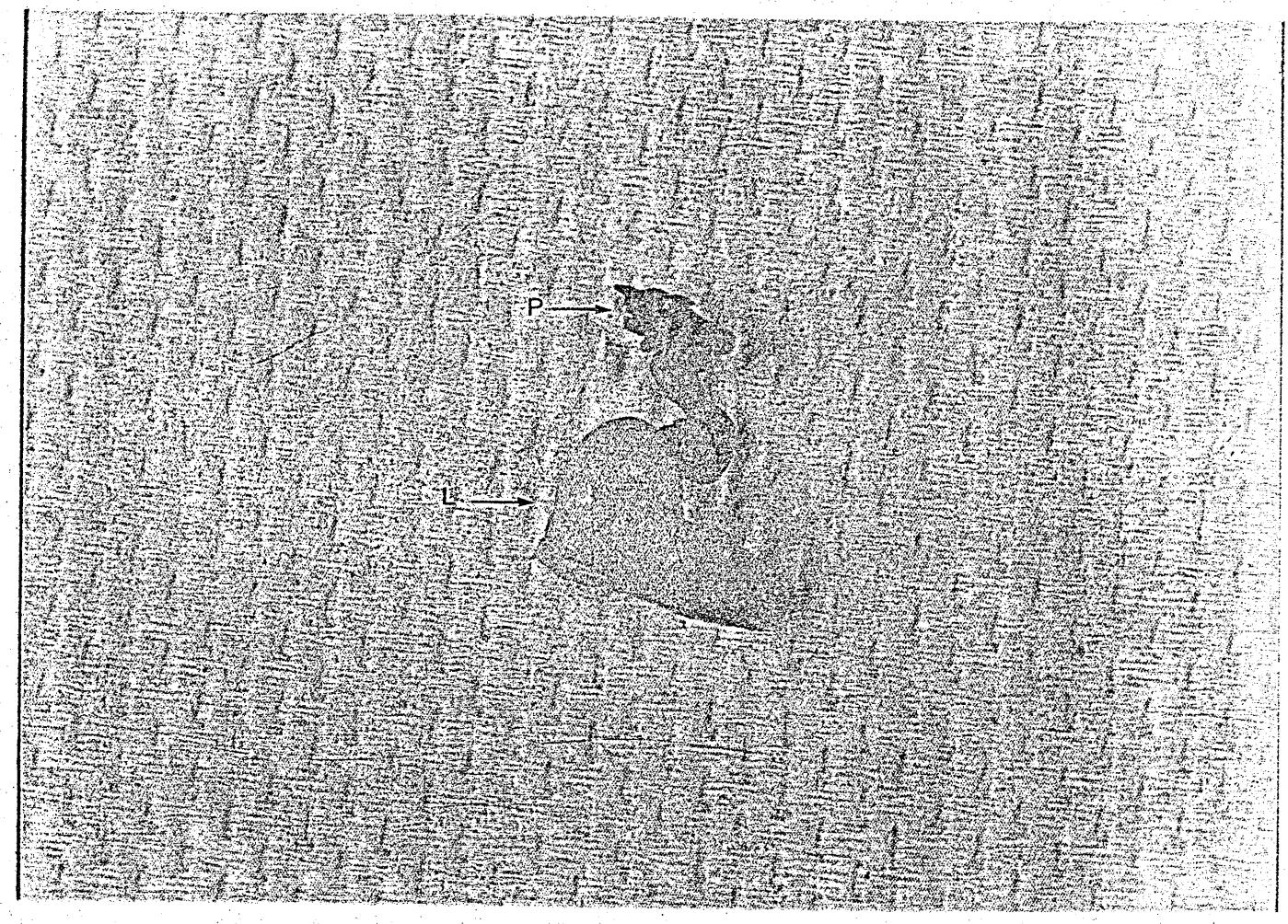


Fig. 3.3:3 A lipoma attached to pancreas from eight months old obese mouse, after seven weeks of three days fasting, one day feeding regime.

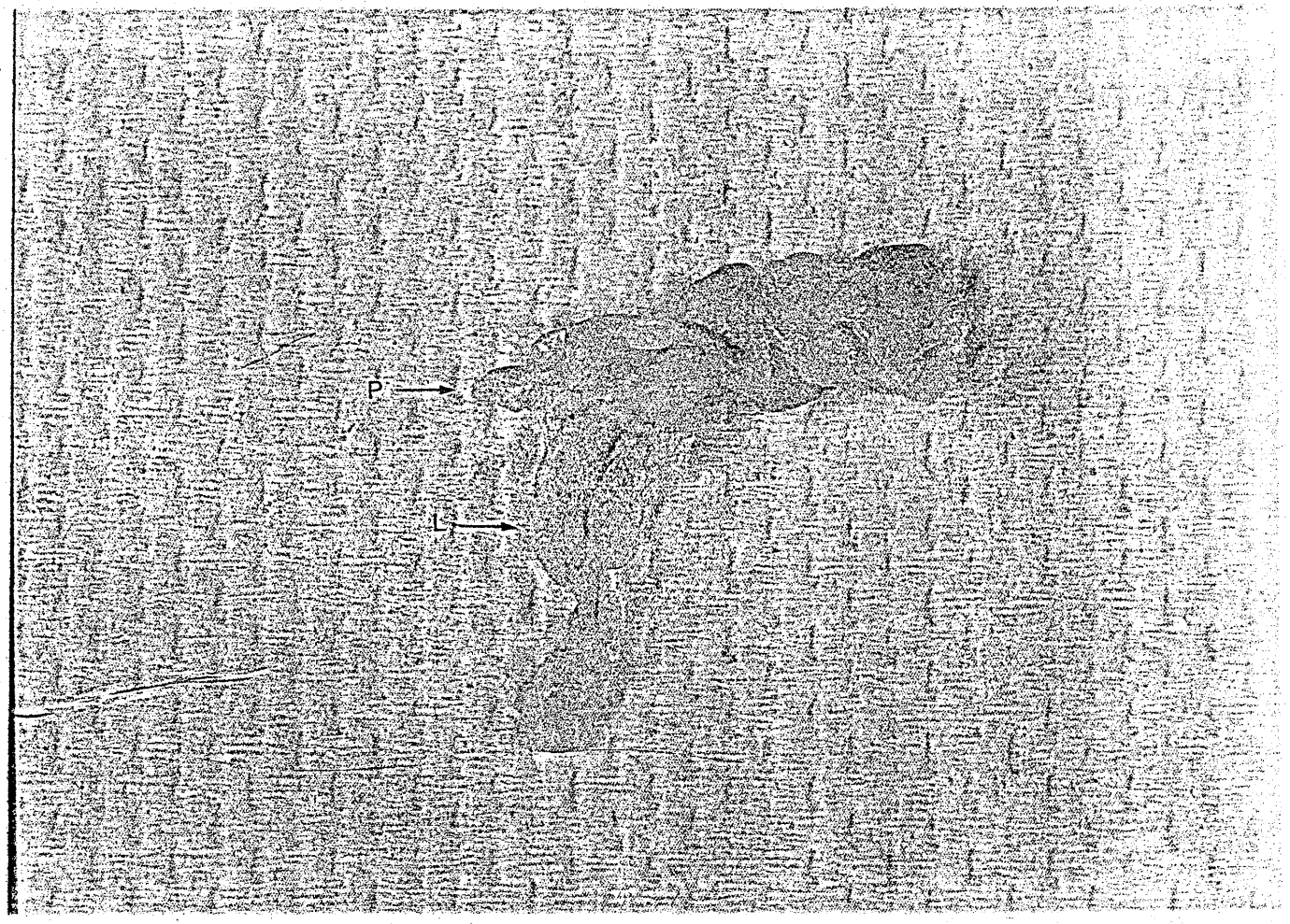


Fig. 3.3:4 A lipoma attached to pancreas from eight months old obese mouse, after seven weeks of three days fasting, one day feeding regime.

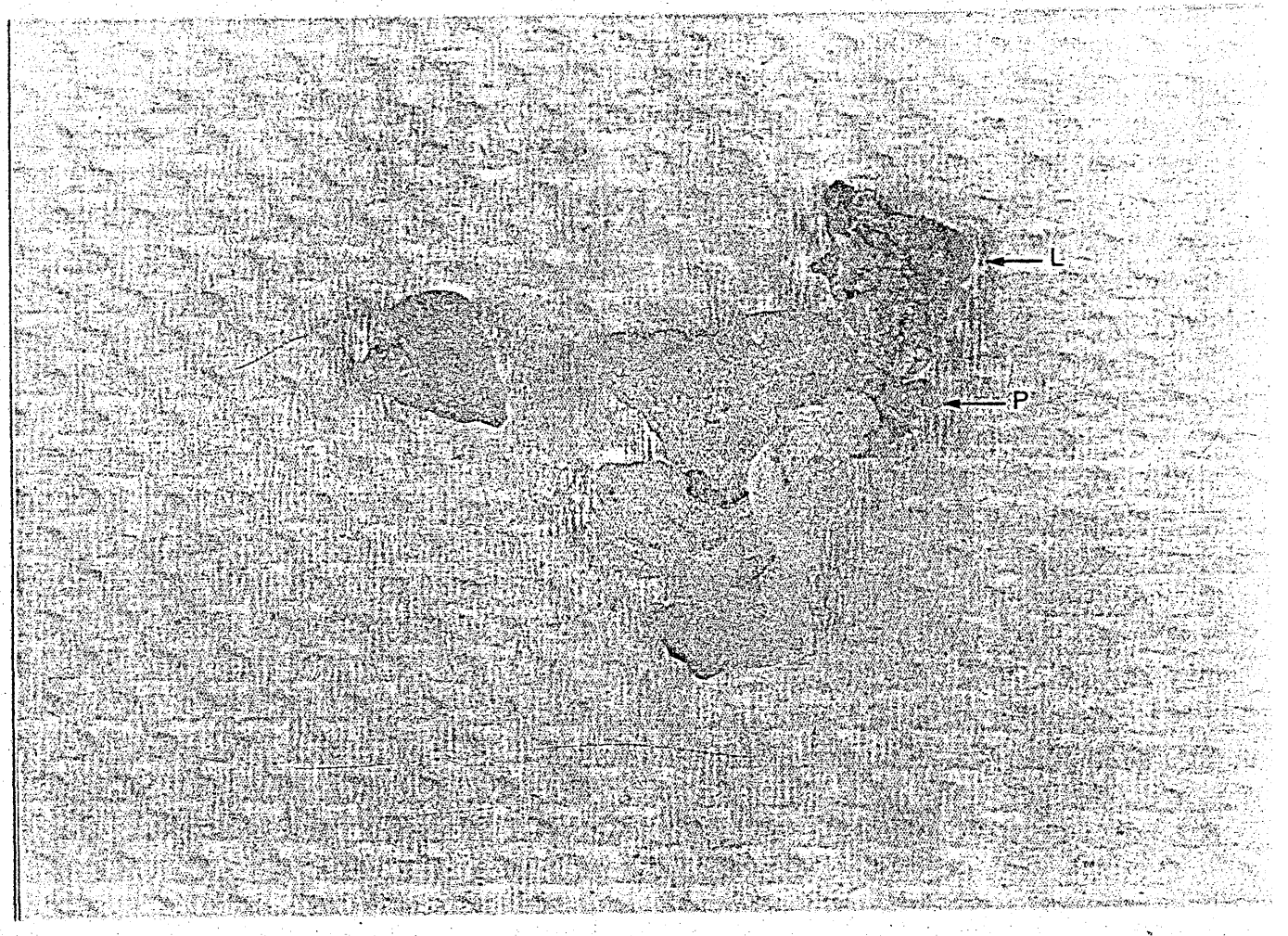


Fig. 3.3:5 A lipoma attached to pancreas from eight months old obese mouse, after seven weeks of three days fasting, one day feeding regime.

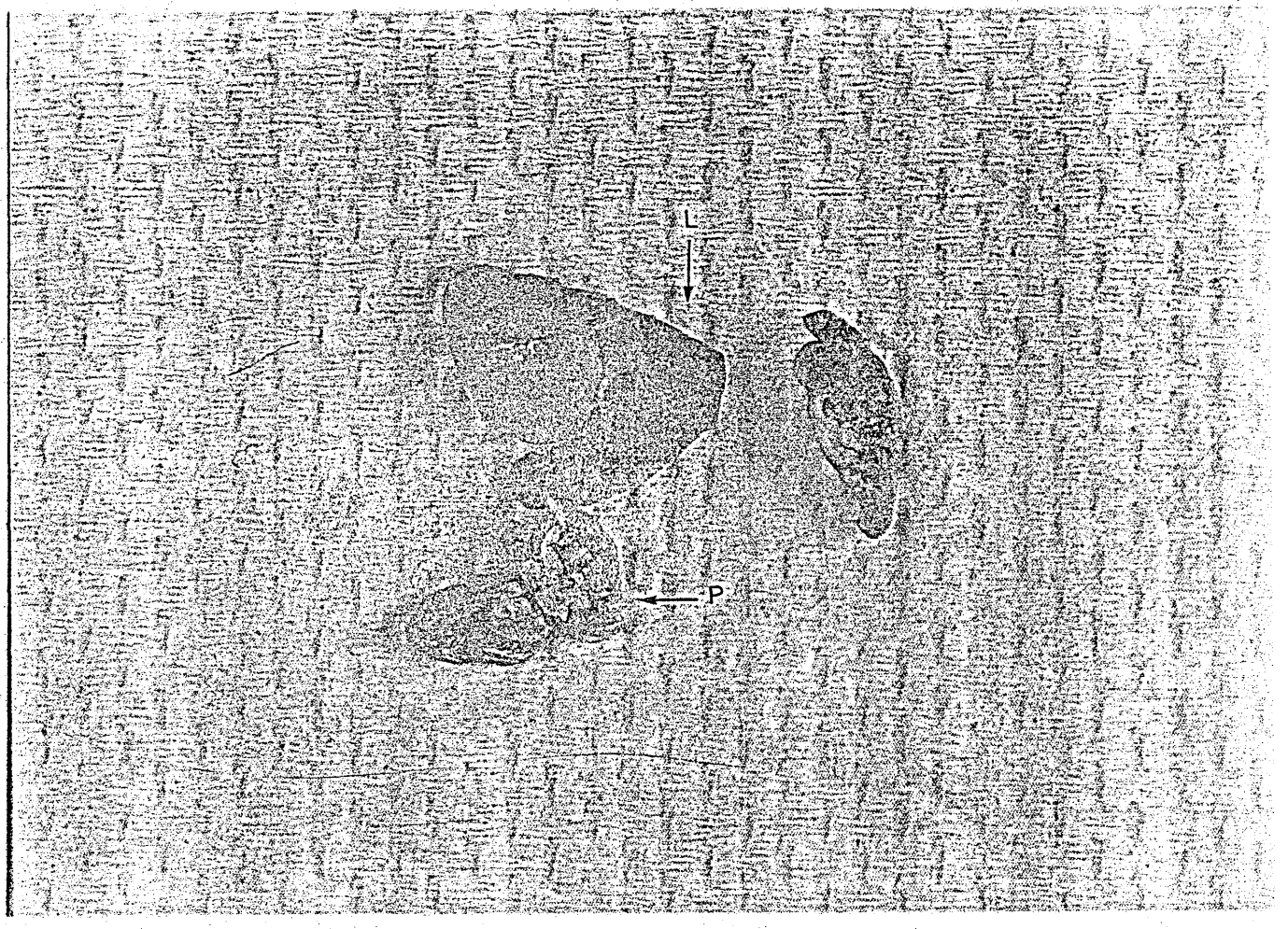


Fig. 3.3:6 A necrotic lipoma attached to pancreas from an eight month old mouse after seven weeks of three days fasting, one day feeding regime.

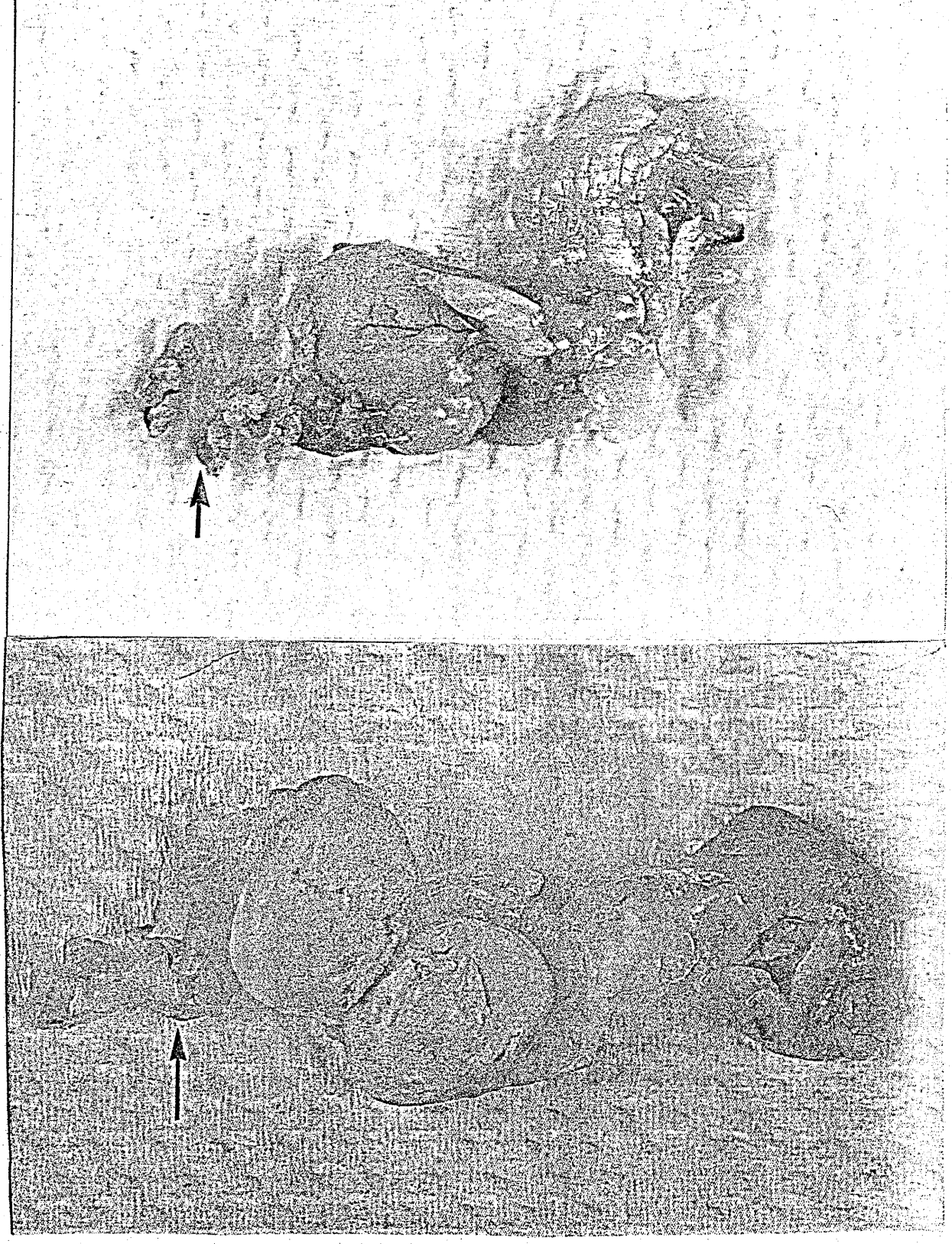


Fig. 3.3:7-8 Large lipoma attached to pancreas (⟶) from obese mouse after seven weeks of three days fasting one day feeding regime. The lower figure shows cut surface of the lipoma.

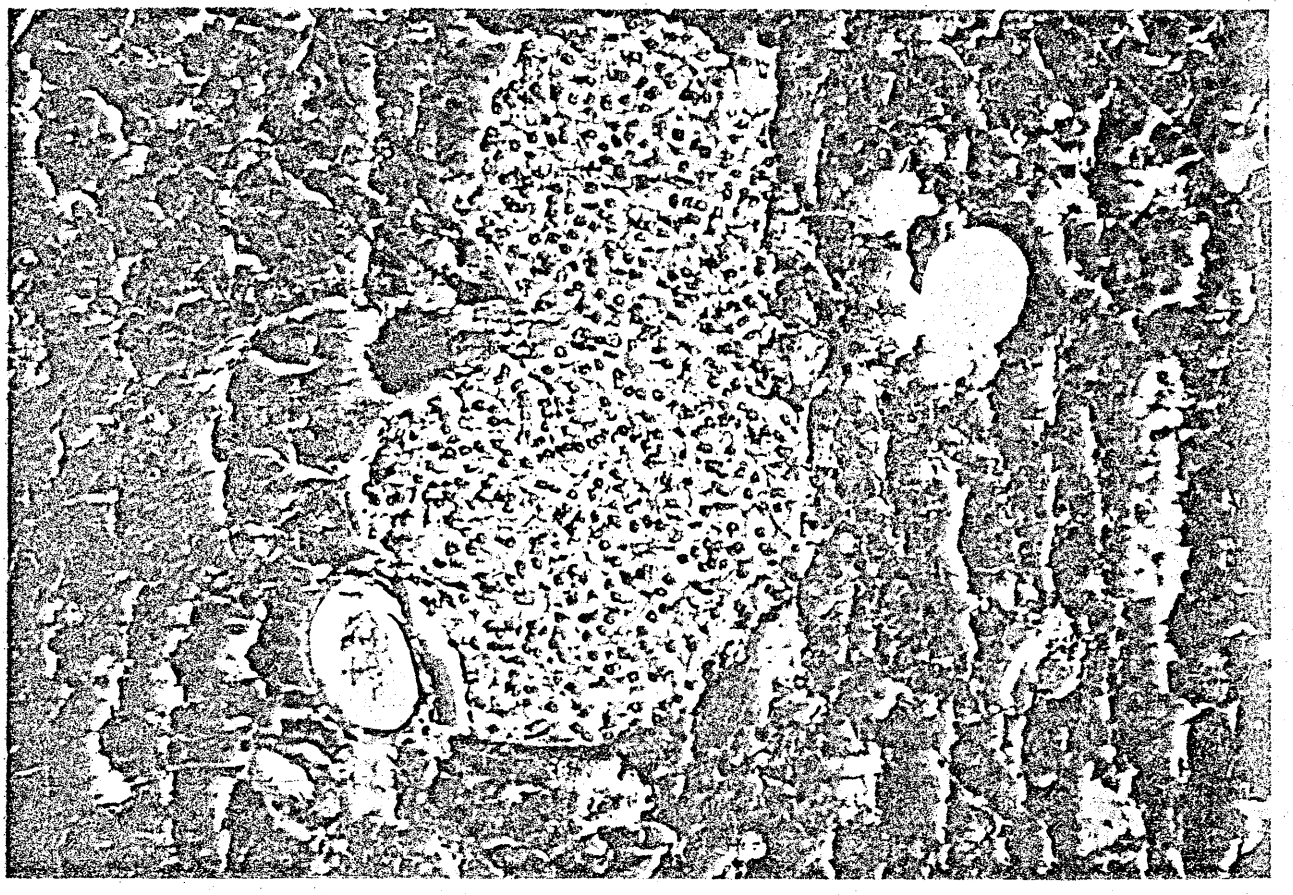


Fig. 3.3:9 Pancreas showing islets, from obese mouse after seven weeks of three days fasting, one day feeding regime. The histological appearance is like that of the control mice (HE X 100).

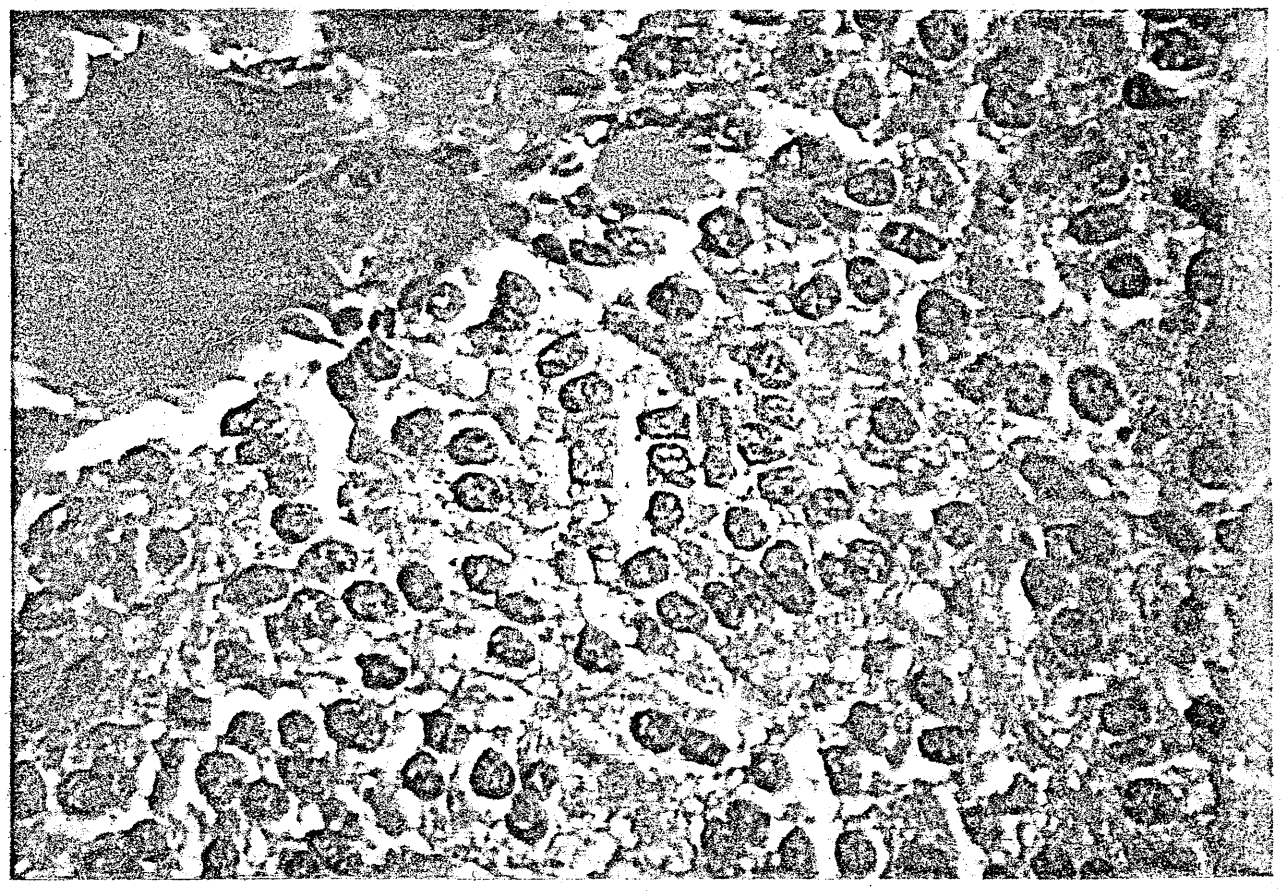


Fig. 3.3:10 High magnification of the islet cells from the previous pancreas (HE X 400).

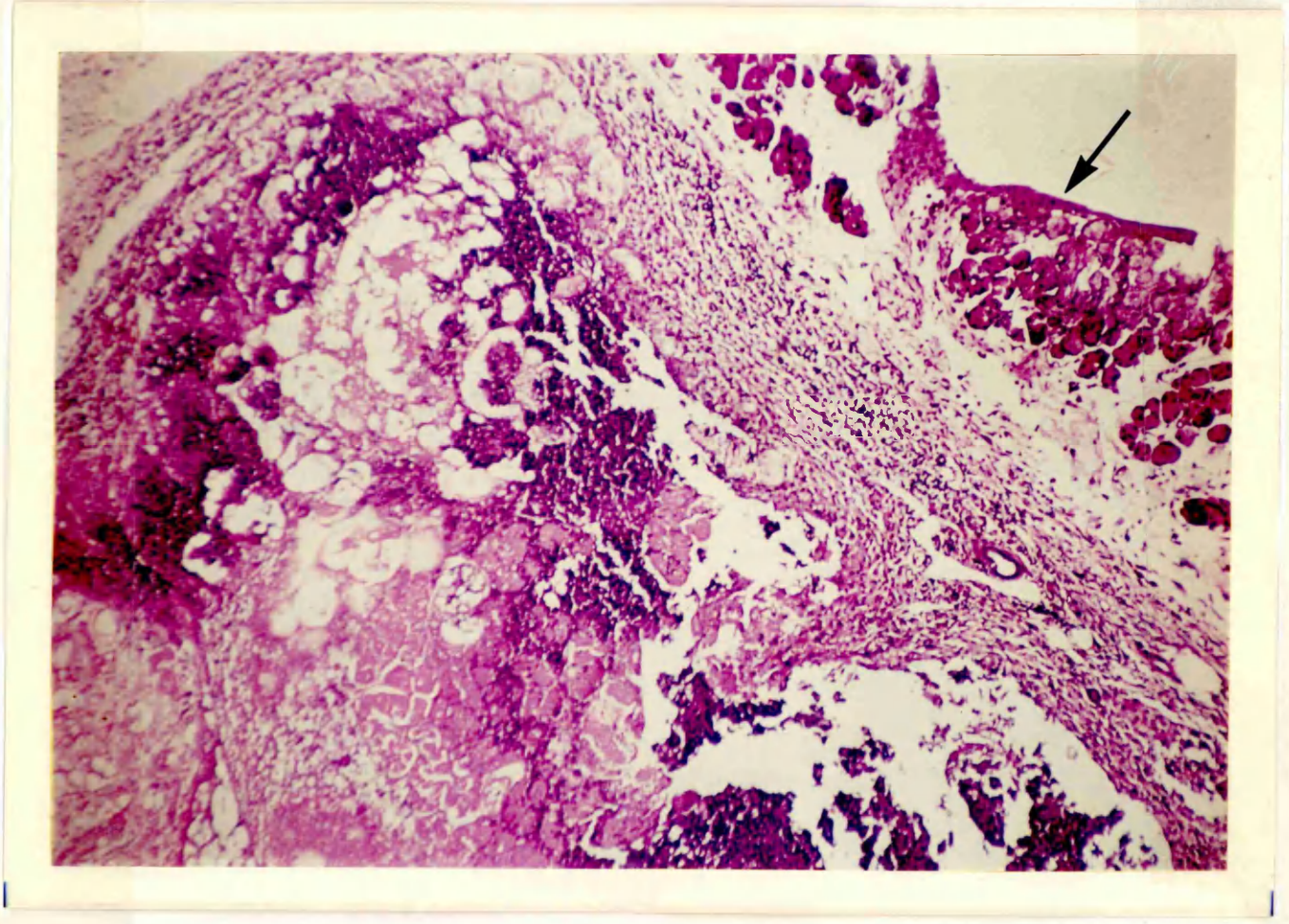


Fig. 3.3:11 Necrotic lipoma with fibrous capsule and inflammatory cell infiltration attached to the pancreas (⟶) (HE X 100).

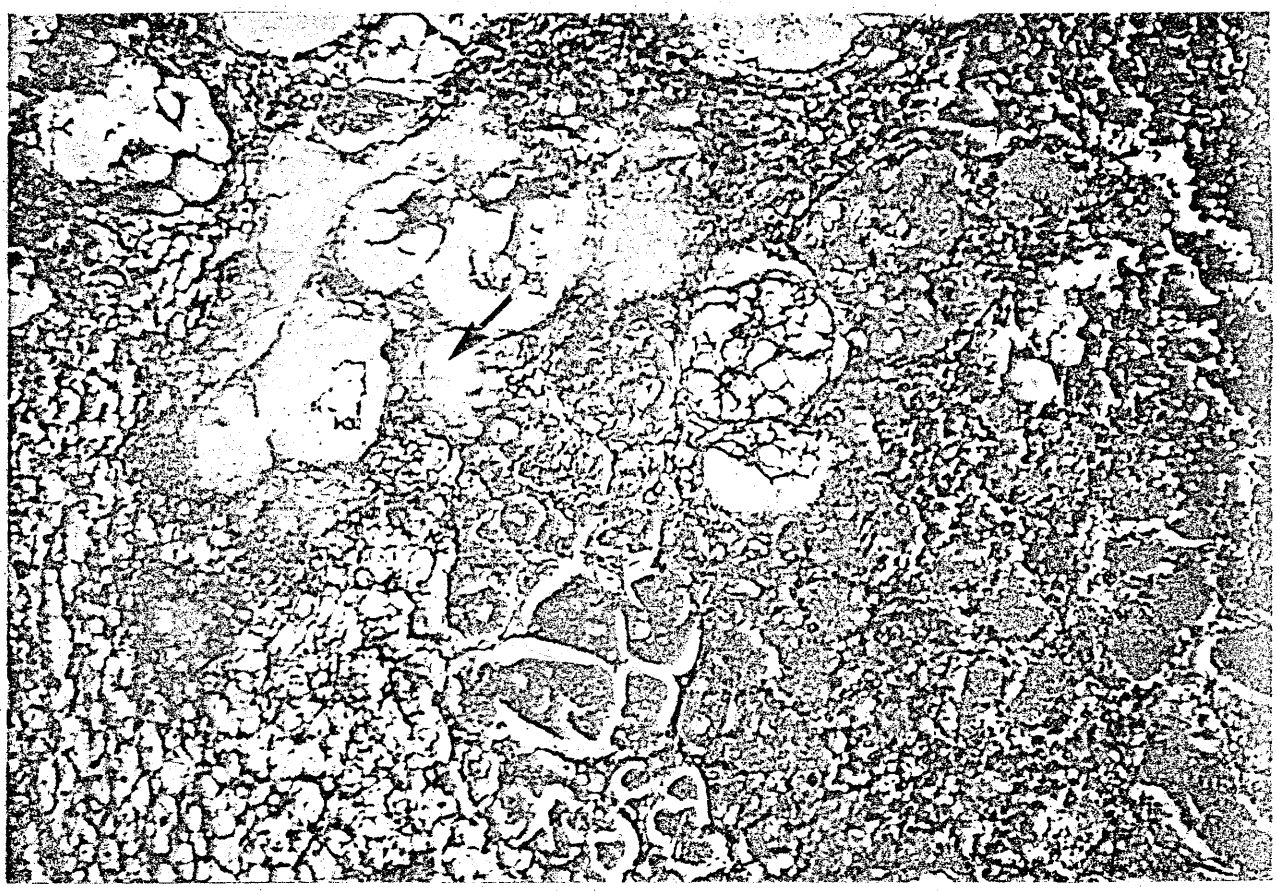


Fig. 3.3:12 Outline of necrotic fat cells (⟶) with inflammatory cell infiltration in another lipoma.

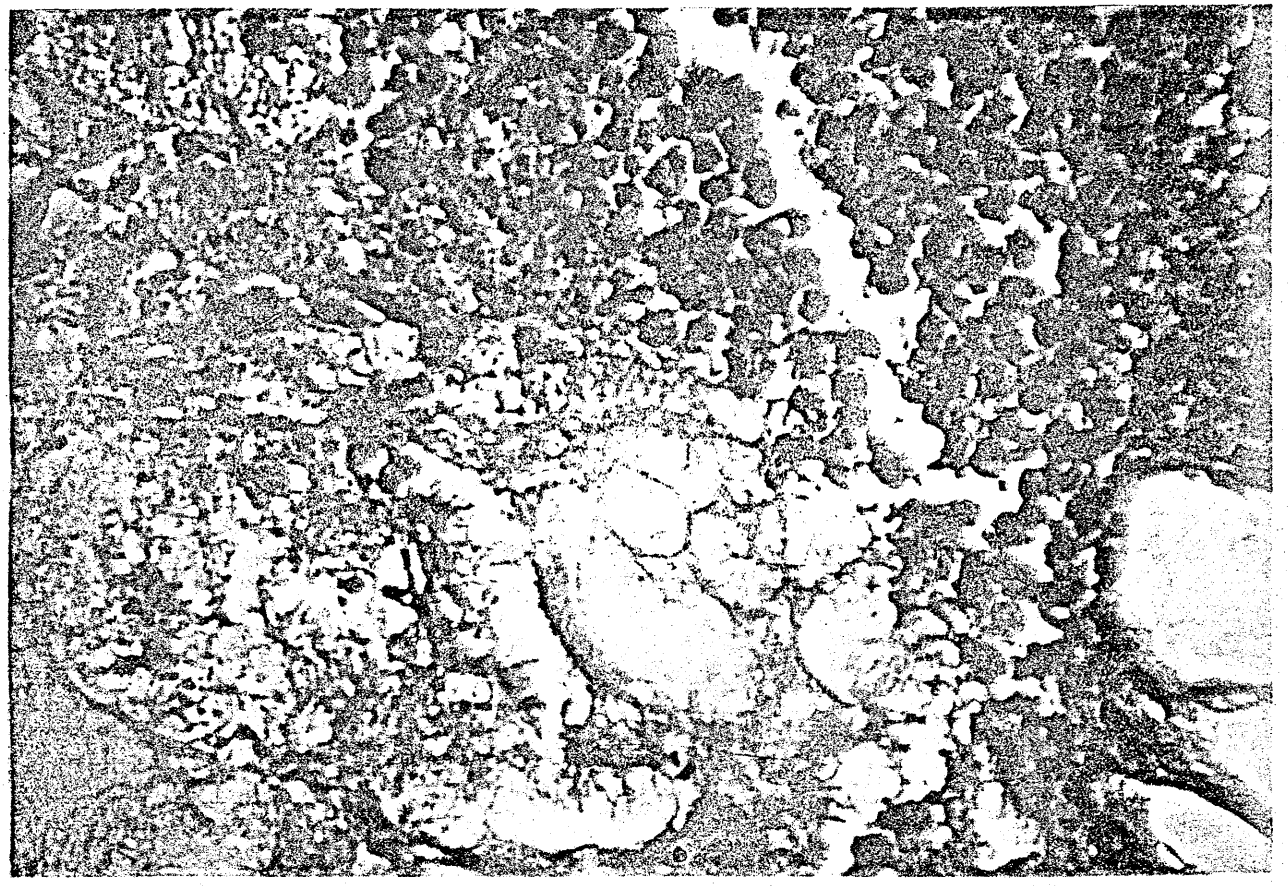


Fig. 3.3:13 High magnification of the previous lipomas showing necrotic fat cells (HE X 400)

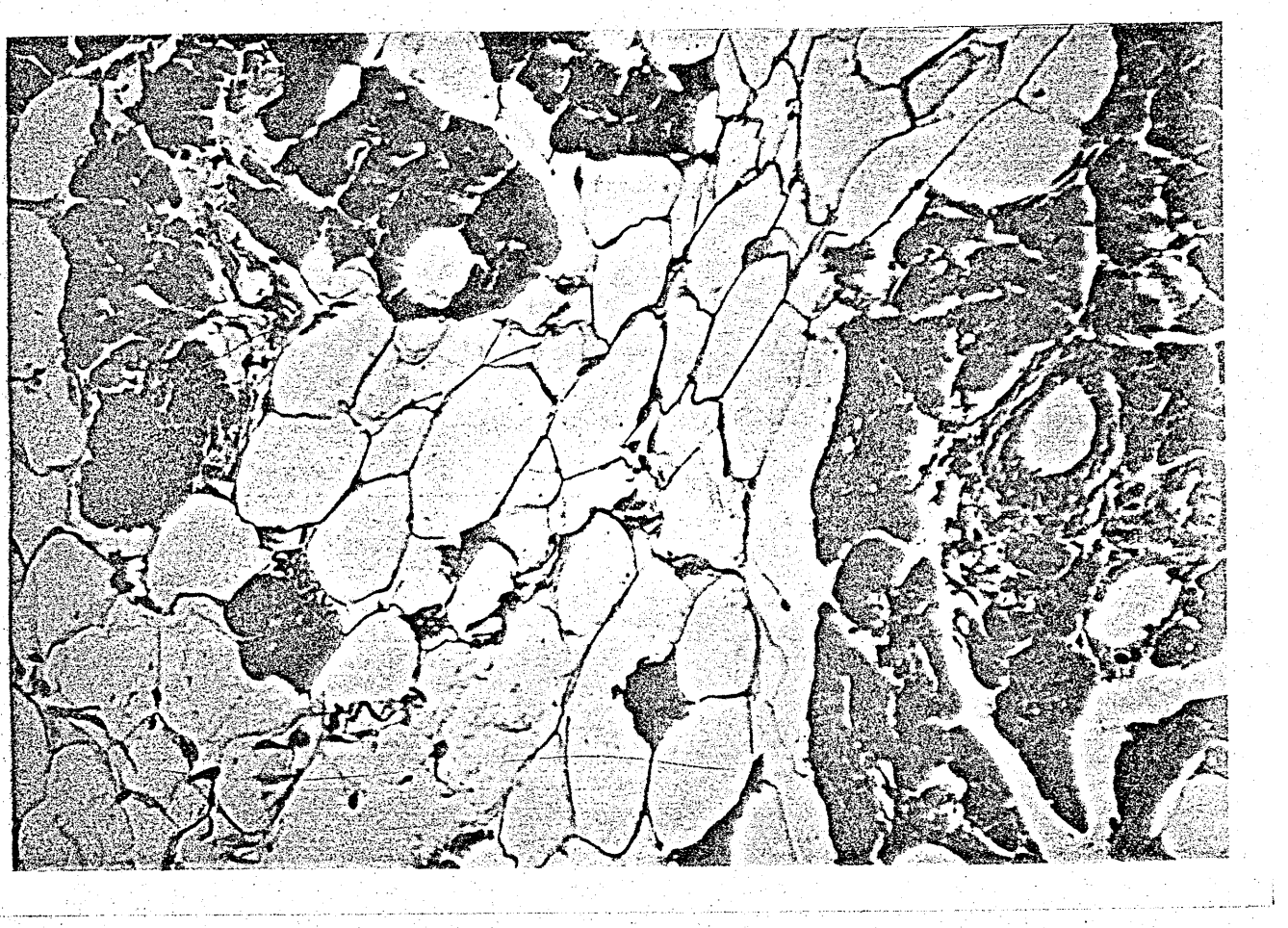


Fig. 3.3:14 Infiltration of fat cells into the pancreas of obese mice after seven weeks of three days fasting, one day feeding regime (HE X 100)

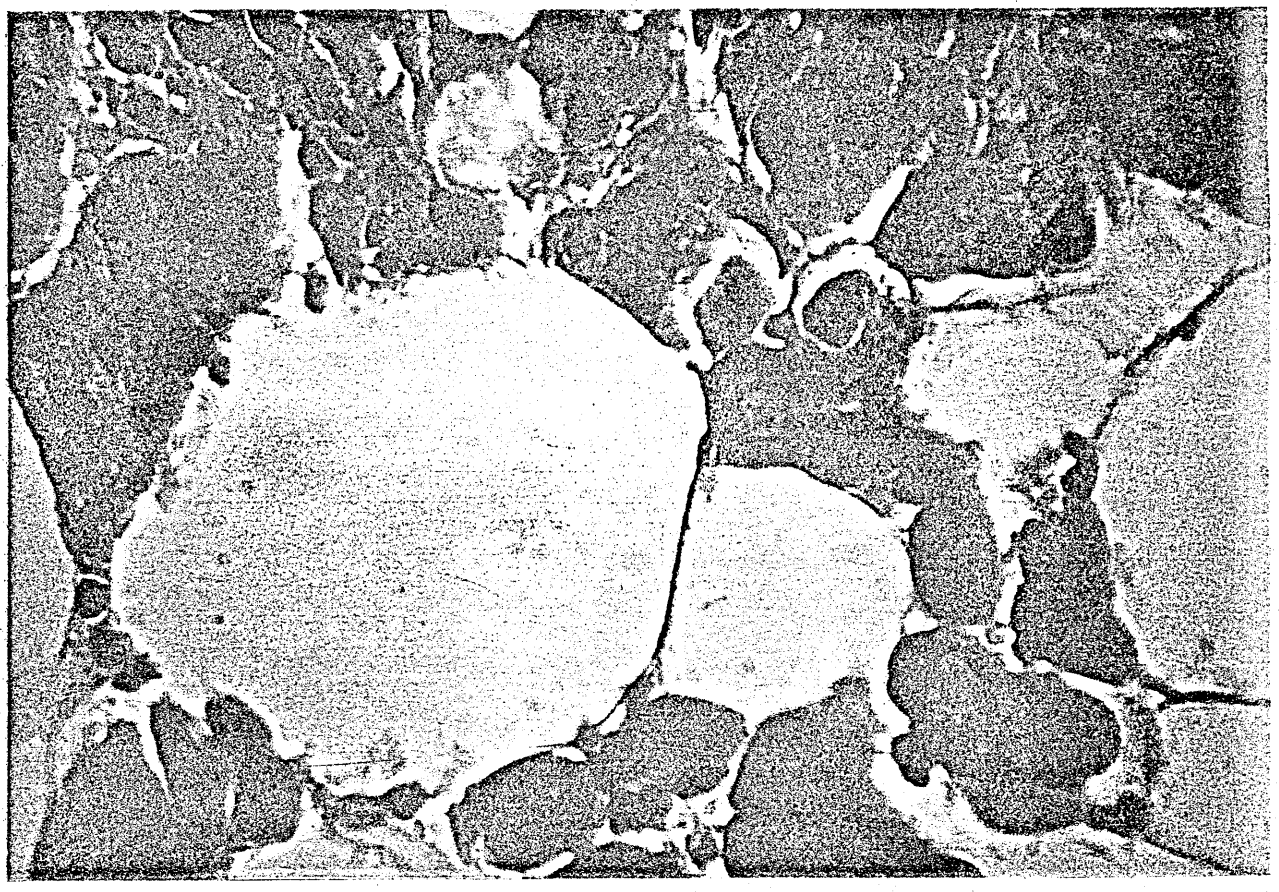


Fig. 3.3:15 High magnification of exocrine pancreas with infiltrated fat cells from the previous mice. (HE X 400)

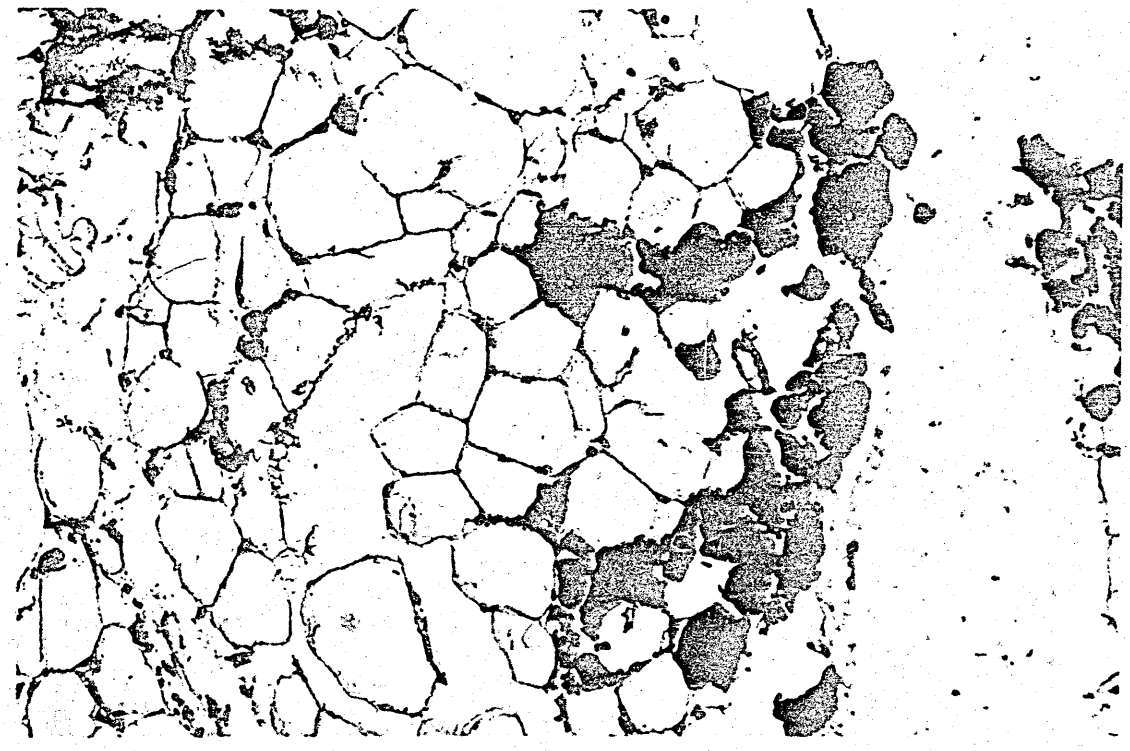


Fig. 3.3:16 Infiltration of fat cells into the pancreas from another obese mouse after seven weeks of three day fasting, one day feeding regime.

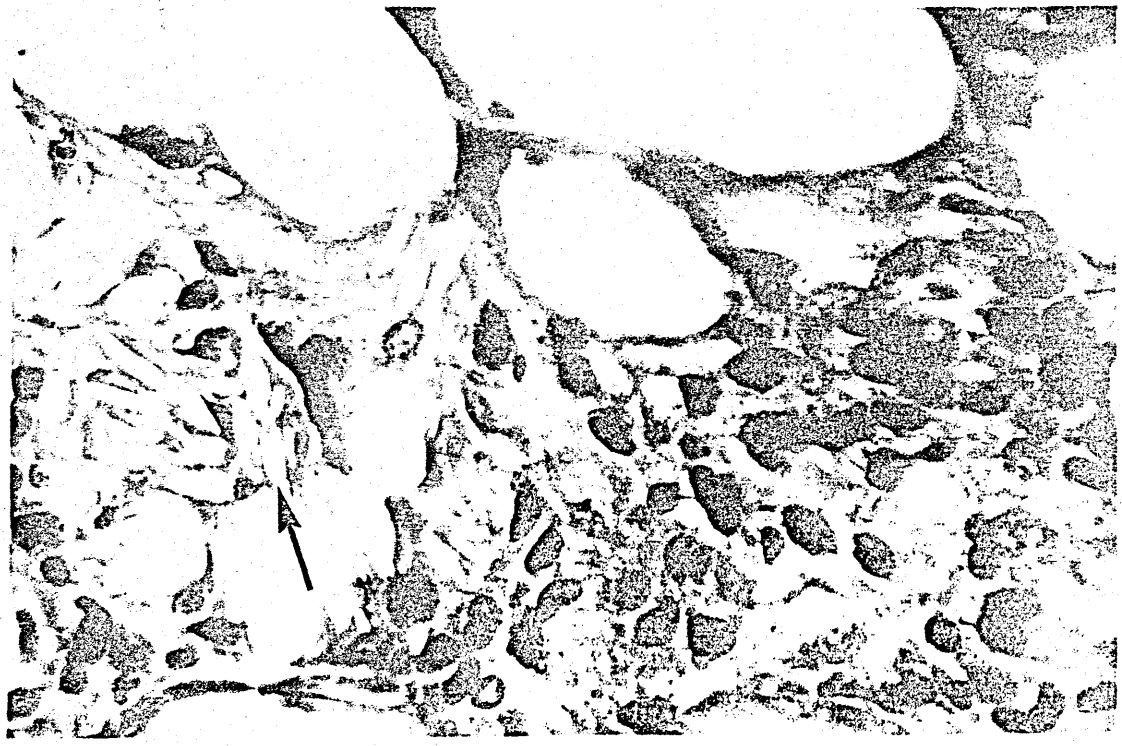


Fig. 3.3:17 High magnification showing focus of cholesterol clefts in a lipoma (HE X 400).

active βcells (Gepts et al 1960; Hellman and Peterson 1960, Hellman et al 1961; Hellman et al 1962; Renold, Orci, Stauffacher, Junod and Rouiller 1970).

Histological examination of the tumours (Fig. 3.3:11-17) confirmed that they were all lipomas, attached to the pancreas. In some cases, fat cells infiltrated the pancreatic tissues (Fig. 3.3:14-17).

2 - Discussion

It is known (Wills 1953, Morson and Dowson 1974) that lipomas occur naturally in mice. Nevertheless, the finding that six out of the twelve ob/ob mice, which were on a weight reducing regime for seven weeks (three days fasting, one day feeding) had lipomas attached to the pancreas in the same way, whilst none of the control mice, whether obese (12) or lean (12) mice had lipomas is highly significant.

It is not known whether lipomas were present in the other obese mice groups which had their weight reduced in the previous experiments (Section 3.2) as these mice were not dissected.

The presence of lipomas in obese hyperglycaemic mice is seen from time to time, but they are not usually attached to the pancreas, nor with such high incidence (Dr. M. Lancaster - personal communication, 1975).

A possible explanation for this phenomenon was given by Dr. Norman B. Gibbs (Consultant Pathologist, St. Luke's Hospital, Guildford) who suggested that lipomas arise at the tail of the pancreas as a result of pancreatic lipase lackage from the pancreas. Indeed, this is the explanation put forward for occurrence of lipomas in man, following inflammation of the pancreas (acute pancreatitis). It is now well recognised that pancreatic lipase attacks the adipose tissues around the pancreas causing fat necrosis and/or a rise to lipomas but, in my opinion, this still does not explain the mechanism of neoplastic transformation.

Whether alternate fasting and feeding regime accelerate or precipitate the lackage of pancreatic lipase from the pancreas, should be investigated in further studies.

3.4 The Possible Role of Gut Hormones in the Development of Obesity in Obese Hyperglycaemic Mice (ob/ob)

Preparation of Materials for Histological and Immunohistochemical Experiments

Two sets of twenty obese hyperglycaemic mice aged four and eight months together with their littermates were chosen for this study. The choice of the two age groups was made following the observation of body weight changes of the mice with age as seen in Fig. 3.4:1.

By the time the experiment had begun, the older set of obese mice (eight months), had already reached their maximum weight, but the young mice (four months) were still gaining weight.

The two test groups of obese mice (ten mice per group) were kept for seven weeks on a 72 hours fasting, 24 hours ad libitum feeding regime (as described in Section 3.2). Whereas the matching lean (twenty mice) and obese controls (twenty mice) were fed ad libitum throughout. Body weight changes in each group at the end of this experiment are summarised in Table 3.4:I.

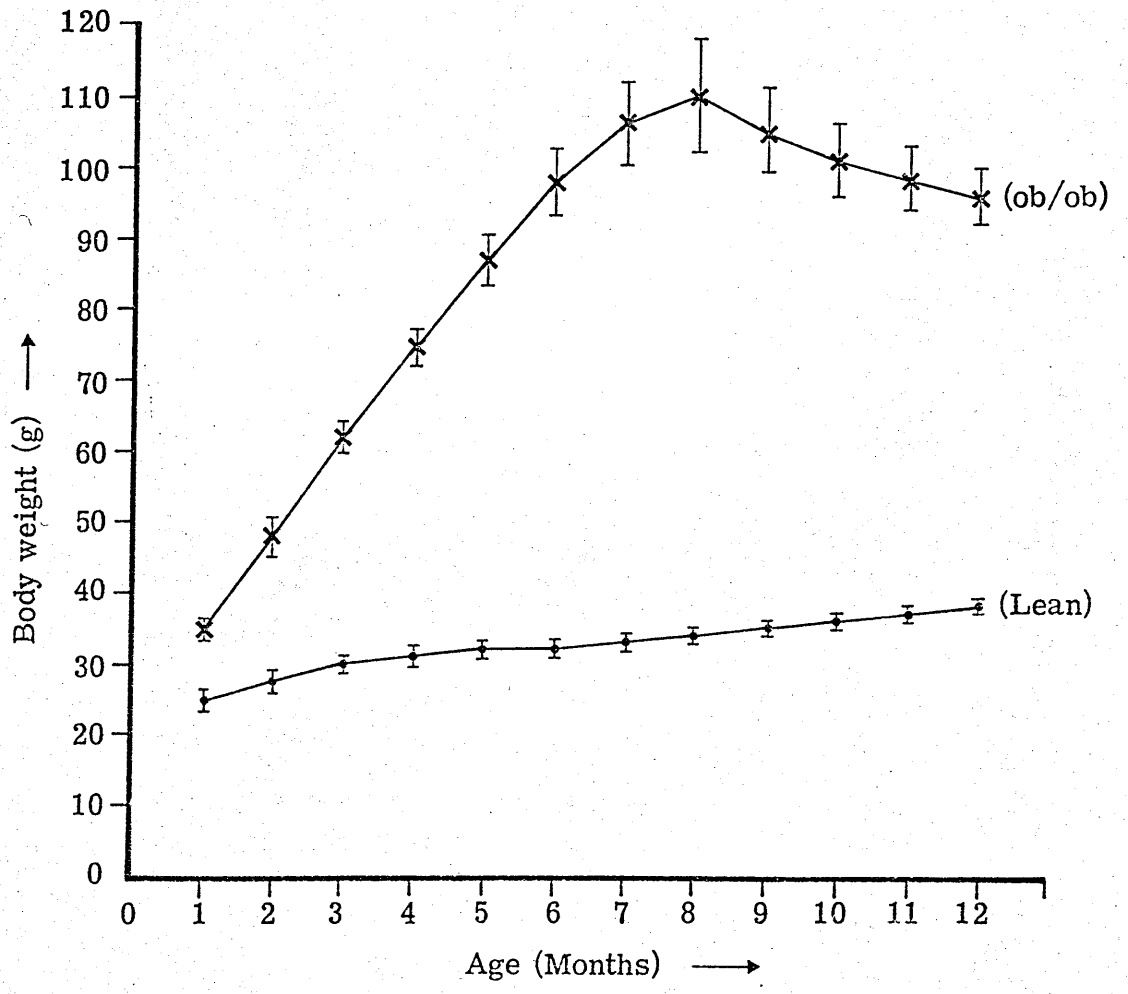
Mice	Group	No.	Mean Body Weight (g)		% Weight Changes
			0	7 weeks	
Young obese	Test	10	91	47	-48.6
(4 months)	Controls	10	95	97	+ 2.0
Young lean	Controls	10	34	35	+ 2.8
Old obese	Test	10	99	88	-11.1
(8 months)	Controls	10	110	59	-47.5
Old lean	Controls	10	36	38	+ 5.2

- loss: + gain

Table 3.4:I

Body weight changes of obese mice after seven weeks of 72 hours fasting, 24 hours ad libitum feeding regime.

Fig. 3. 4:1 Mean(± SEM) body weight of 30 obese hyperglycaemic mice (ob/ob) and 30 lean mice of different ages, fed rat chow diet ad libitum



At the end of the weight reduction period, the mice were killed, after an overnight fast, by cervial dislocation and tissues were taken from the antrum, pancreas, 7 cms of proximal duodenum, followed by the adjacent 7 cms of the upper jejunum. Then, missing about 5-7 cms length, a further 7 cms were taken from the lower jejunum. A similar procedure was followed along the gut as far as the proximal colon.

Flat pieces of mucosa from each area were quenched in 'Arcton' (Freon-22) at -156^{o}C each piece was kept in a plastic bag, labelled and kept in flasks containing solid CO_2.

The materials were then sent in flasks (containing solid CO_2) to Dr. J. Polak at the Department of Histochemistry, Royal Postgraduate Medical School, London. She kindly undertook to study the possible changes in the gut hormone producing cells in these mice as a result of weight and insulin reduction by means of quantitative histology of silver stained preparation which demonstrates the majority of endocrine cells and quantitative immunocytochemical studies by image analyser which determine the number and hormone content of the endocrine cells in the gut.

These techniques are described in detail by Pearse 1969; Pearse, Polak, Adams and Kendal 1974; Pearse and Polak 1975; Polak, Pearse, Adams and Garaud 1974; Polak, Pearse, Garaud and Bloom 1974; Polak, Pearse and Heath 1975b.

The histological and immunocytochemical studies described are still incomplete.

3.5: Discussion

Body weight, blood glucose and plasma insulin levels of obese hyperglycaemic mice (ob/ob) were all markedly reduced, in a relatively short time, by alternate periods of fasting for 72 or 48 hours and refeeding for 24 hours. Physical activity increased significantly with the decrease in weight. The mice regained weight on the resumption of normal feeding and the blood glucose and plasma insulin concentrations rose rapidly to their original levels.

Breeding from 'slim' ob/ob mice was not possible. This finding is in contrast to the report by Lane and Dickie (1954), who used different slimming regime. The results suggest that failure to breed even from 'slim' ob/ob mice, is not due to obesity as such, but to another primary abnormality, causing irregular and infrequent cycling periods in the female ob/ob mouse, and marked reduction volume and nuclar size of Leydig cells in the male mouse (Coleman and Hummel 1968; Lidell and Hellman 1966; Hellman, Jacobsen and Taljedal 1963).

The finding of a significant increased incidence of lipomas (some with necrosis) attached to the tail of the pancreas, in ob/ob mice after seven weeks on a weight reduction regime of 72 hours fasting, 24 hours ad libitum feeding (Section 3.3) was not expected and should be further investigated

It is possible that the alternate fasting and refeeding regime which caused changes in blood glucose and plasma insulin levels could also cause leakage of pancreatic lipase from the pancreas giving rise to fat necrosis and lipomas at the tail of the pancreas.

Fatty infiltration of the pancreas, with an increase in the amount of intestinal fat, is common in cases of human obesity (Anderson 1961). However, this is not usually associated with lipomas.

Whether alternate fasting and refeeding regimes accelerate or precipitate the leakage of the pancreatic lipase from the pancreas, should be investigated in further studies.

Certainly the alternate fasting and refeeding regime employed in the present study were well tolerated by the obese mice and were effective in producing marked and progressive weight loss without apparent ill effect.

Treatment with varying doses of triiodothyronine (T_3) failed to cause significant weight loss in ob/ob mice with free access to food. This is consistent with the finding of normal thyroid status in ob/ob mice (Goldberg and Mayer 1952; Wykes et al 1958; Bray and York 1971).

The suggestion by Joosten and Van der Kroom (1974), that the obesity of the ob/ob mice is due to hypothyroidism receives no support from the present work. This does not, however, preclude the possible involvement of other hormonal factors.

The rapid increase in overnight fasting blood glucose and plasma insulin during normal feeding of 'slim' ob/ob mice (8-11 weeks Section 3.2) suggests that both are secondary manifestation of a primary cause (hyperphagia), which is responsible for the obese hyperglycaemic syndrome.

The possibility has been entertained in the past that excessive secretion of insulin, due to an intrinsic abnormality of β-cell function, might be the initial cause of obesity in ob/ob mice. Excessive secretion of insulin has also been held to be responsible for some cases of human obesity (Marks and Samols 1968).

A serious objection to any hypothesis assigning a causative role to hyperinsulinaemia due to a primary β-cell abnormality, however, is the fact that dietary induced weight loss both in human subjects and experimental animals is accompanied by a rapid fall in plasma insulin levels. These may reach normal values - both in the fasting and fed state - long before normal weight has been restored and whilst substantial adiposity is still present.

This latter observation makes it equally difficult to explain an alternative theory linking obesity with hyper-

insulinism, namely that obesity causes insulin resistance, and hence the necessity to secrete excessive amounts of insulin to maintain normal glucose homeostasis. Whilst both hypotheses may be correct - in some cases - the third possibility that both obesity and hyperinsulinism are a consequence ofthe same primary cause - namely hyper-alimentation with consequent over-stimulation of the intestinal insulin release factors - has until recently received little attention (Fig. 3.5:1).

Evidence that gut insulin releasing factors can exert a priming effect upon the β-cells of the pancreas, as well as stimulating them to secrete insulin directly, has been produced by Turner and Young (1973). Moreover, the hypothesis that primary over-production of intestinal hormones is responsible for the hyperinsulinaemia is consistent with evidence recently obtained (Polak et al 1975b), that the intestinal endocrine cells, shown to be capable of secreting insulin releasing polypeptide hormones, gastric inhibitory polypeptide (GIP) and glucagon-like immunoreactivity (GLI), are greatly increased in number in ob/ob mice compared with normal weight (lean) litterates.

Whether a prolonged period of hypoalimentation produces a reduction in the intestinal endocrine cells and so accounts for the reduction in basal and glucose stimulated insulin release from the β-cells is currently under investigation (Section 3.4).

The amino acids sequence of intestinal insulin releasing polypeptide (IRP) is not known, since IRP has yet to be isolated in a pure enough form. Although IRP preparations did not significantly cross-react with anti GIP-antibody (Turner et al 1974 a,b) this does not preclude the possibility that IRP preparations are contaminated with GIP, which may be responsible for the insulin releasing activity of the crude IRP preparations.

The hypothesis, suggested by Professor V. Marks linking gut hormones, insulin secretion and obesity, which is summarised in Figs. 3.5:1-3, appear at present to be a possible mechanism in the development of obesity.

HYPOTHESIS FOR DEVELOPMENT OF OBESITY AND SEQUENCE OF MECHANISM*

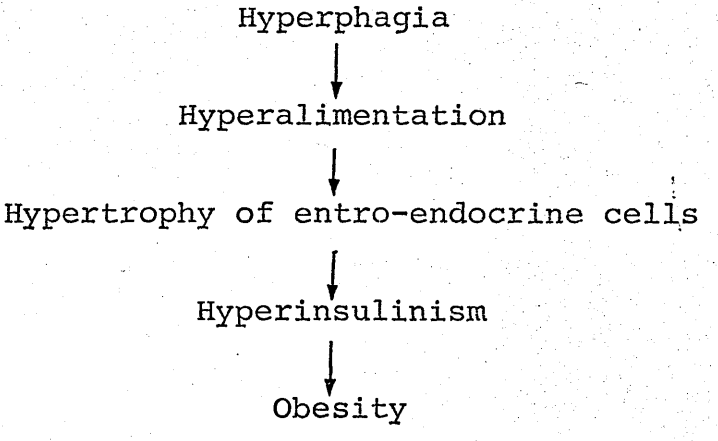


Fig. 3.5:1

*Courtesy of Professor V. Marks

POSSIBLE INVOLVEMENT OF GUT HORMONES GIP, GLI AND IRP IN PATHOGENESIS OF OBESITY (HYPOTHETICAL)*

1. Over-production of GIP, GLI and/or IRP in the cause of insulin over-production.

   (i) by direct action of β-cells
   (ii) priming effect on β-cells.

2. Hypersecretion of gut hormones is due to

   (a) excessive stimulation of gut hormones, secondary to high concentration of IRP, GIP releasing sugars in human gut
   (b) inherited hypersensitivity of gut hormones releasing mechanism.

Fig. 3.5:2

*After Professor V. Marks

HYPOTHESIS LINKING INSULIN SECRETION AND OBESITY*

1. Increased insulin secretion is the prime mover and key factor in the pathogenesis of 'some cases of' human obesity.

2. The increase in insulin secretion is itself secondary to increased secretion of intestinal hormones including the intestinal insulin releasing polypeptide (IRP) described by Turner, gastric inhibitory polypeptide (GIP) described by Brown and gut glucagon cross reacting materials (glucagon-like immunoreactivity, GLI, enteroglucagon) described by Samols.

Fig. 3.5:3

*After Professor V. Marks

C H A P T E R 4

GENERAL DISCUSSION

The rather close association between obesity, diabetes and hyperlipaemia is well known (Kannel el al 1967). It is thought that the association between obesity and diabetes is caused by peripheral insulin ineffectiveness leading to insulin over production (Perley and Kipnis 1966).

Obese hyperglycaemic mice (ob/ob) provide an interesting model of diabetes, in that they present at the same time, obesity, hyperglycaemia and hyperinsulinaemia. It thus has several characteristics in common with human maturity onset diabetes (Vague and Boyer 1974).

The experiments performed on the obese mice have shed some light on the nature of the mechanisms involved in the development of obese hyperglycaemic syndrome.

The important finding of significant hyperplasia of the GIP cells in the gut of ob/ob mice (Polak et al 1975a) is open to a number of explanations. This hyperplasia may be primary (genetic) or secondary. If the latter case applies, then the following causes should be considered.

1. Increased food intake (Bray and York 1971) with over-stimulation of GIP cells.

2. Increased intestinal absorption of glucose (Mayer and Yannoni 1956).

3. Glucose-mediated release of GIP with overdriving of the cells.

4. Increased synthesis of GIP in response to requirement for increased insulin output to balance high serum glucose levels, since GIP is an insulin releasing hormone (Dupré et al 1973, Turner et al 1974 a, b).

It is possible that another insulin releasing gut polypeptide IRP (Turner et al 1974 a, b; Shabaan et al 1974 a,b) could be concerned with production of the syndrome.

It is hoped that the histochemical studies which have already started to look at the gut of ob/ob mice which had their weight reduced would confirm the role of the gut hormones in the development of obese hyperglycaemic syndrome.

The recent evidence by Heptner and his colleagues (1975) shows that intragastric administration of glibenclamide causes the release of gut hormones effective in stimulating insulin release, suggests that in maturity onset diabetes, the typical pattern in serum insulin following the intake of food components, might be primarily due to a certain defect in the gastrointestinal tract rather than in the β-cells of the pancreas itself.

The alternate fasting and refeeding regimes which were effective in producing rapid and progressive weight loss in ob/ob mice, might also form an acceptable basis for dietary therapy in some forms of human obesity. Duncan and his colleagues (1962, 1963) have used an initial period of fasting for a week or two, followed by one or two day fasts, to maintain weight loss or promote further weight loss. In view of the practical difficulties encountered in the treatment of obese subjects by long periods of complete starvation, the alternate 48 hours fasting, 24 hours feeding regime might be an easier alternative.

Obese hyperglycaemic mice (ob/ob) and hyperlipaemic rats, induced by cobalt chloride have been proven to be useful experimental models in studying obesity and hyperlipaemia.

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A P P E N D I X I

SUPPLIERS OF MEDIA, MEDIA SUPPLEMENTS

CHEMICALS AND RADIOCHEMICALS

Materials	Suppliers
Calf Serum	Biocult - Paisley, Scotland.
Cobalt (^{57}CO) - Sp.Act. 50 mci/µg Co	
Code: CTS-1:	Radiochemical Centre - Amersham
Cobalt Chloride $CoCl_2 6H_2O$	Kock-light - Buckinghamshire
Collagenase (Type I & II)	Sigma - London
Falcon Plastic Culture Flasks	Scientific Suppliers
Hexane	BDH - Poole
Horse Serum	Biocult - Scotland
Insulin - (20.I.U./ml)	Weddel Pharm. - London
L-15 Culture Medium	Flow Laboratory - Scotland
MEM (Minimum Essential Medium)	Biocult - Scotland
MEM with Earle's BSS	Biocult - Scotland
MEM Hanks BSS and Hepes Buffer	Biocult - Scotland
Methyl Isobutal Ketone (MIBK)	BDH - Poole
Phenoparbitone Sodium (Nembutal^{R})	Abbott Laboratory
P.P.O. (2-5-diphenylozazole)	Packard Inst. Comp. Ill. USA
POPOP	Packard Inst. Comp. Ill. USA
Toluene - Sulphur Free	BDH - Poole
Triiodothyronine (T_3)	Glaxo Res. Lab. - Greenford
Triton X-100	Kock Light Lab.

A P P E N D I X I I

LIST OF PREVIOUS PUBLICATIONS

1. Studies on the Isolation and Characterisation of Intestinal Insulin Releasing Polypeptide. M.Sc. Thesis, University of Surrey (1973).

2. The Effect of an Intestinal Polypeptide Fraction on Insulin Release in the Rat *In Vitro* and *In Vivo*. Endocrinology, 93, (6), 1323-1328 (1973).

3. The Effect of Adrenaline on Insulin Releasing Polypeptide (IRP) Mediated Insulin Release *In Vivo* in the Rat. Diabetologia, 10, 475-478 (1974).

4. Insulin Release in Responses to Intravenous Infusion of Insulin Releasing Polypeptide (IRP) in the Rat. Diabetes, 23 (11), 902-904 (1974).

5. The Role of the Pancreas in Hyperlipaemia in Rats. Diabetologia, 11, (4), 376 (1975).

6. Fibrosarcomas Induced by Cobalt in Rats. Laboratory Animals - 1976 - In press.

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The Effect of an Intestinal Polypeptide Fraction on Insulin Release in the Rat *in Vitro* and *in Vivo*

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ABSTRACT. The effect of insulin release in the rat in response to a partially purified intestinal polypeptide fraction, "Intestinal Insulin Releasing Polypeptide (IRP)," has been studied *in vivo* and *in vitro*. It potentiated the release of insulin in response to 16.7 mM glucose and 16.7 mM leucine in rat pancreas pieces incubated *in vitro*, but had no effect on insulin release in the presence of 3.3 mM glucose.

IRP produced a small increase in the insulin secretory response to the intravenous administration of an amino acid mixture, but the effect was not statistically significant.

A mixture of secretin and pancreozymin, 1 U/kg of each, potentiated the plasma insulin response to intravenous glucose, but to a smaller degree than IRP and did not accelerate glucose disappearance. Secretin studied in a similar manner showed no insulin releasing effect at doses up to 2 U/kg.

IRP contains less than 4 U/mg secretin activity; no detectable pancreozymin or cholecystokinin activity and no significant contamination by GLI. It is concluded, therefore, that the insulin releasing activity of this preparation is not due to any of these factors—separately or in combination—but to a previously uncharacterised polypeptide now isolated from the small intestine. (*Endocrinology* **93:** 1323, 1973)

AN INTESTINAL hormonal mechanism has been proposed for the regulation of insulin release following oral glucose administration (1). It has been suggested that this hormonal activity is not attributable to the known intestinal polypeptide hormones secretin or pancreozymin, or to the glucagon-like immunoreactive material found in the gastrointestinal tract (2).

A polypeptide fraction to which the name "Intestinal Insulin Releasing Polypeptide (IRP)" has been given, has been isolated from porcine duodenojejunal mucosa (3). This preparation, when injected at the same time as an intravenous glucose load resulted in a marked enhancement of both the plasma insulin response and glucose disappearance in the rat (4). No effect was observed when it was administered with an intravenous injection of physiological saline solution. Further studies of the insulin releasing effects of IRP *in vitro* and *in vivo* are reported here.

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Materials and Methods

Intestinal insulin-releasing polypeptide, IRP

The partially purified intestinal extract, IRP, was prepared by the acid ethanol extraction of frozen porcine duodenojejunal mucosa.

The initial extraction was based on the procedure used for the preparation of material with glucagon-like biological activity from human intestine (5).

Frozen porcine duodenojejunal mucosa was extracted with acid ethanol (ethanol, water, conc. HA. 75:25:1.5), 5 ml per g wet tissue. After homogenizing in a Waring blender the mixture was kept for 2 hr at 4 C and then centrifuged at 2,000 rpm for 15 min. The supernatant was retained and the pellets re-extracted with 3 ml/g of starting material and kept overnight at 4 C. The mixture was centrifuged at 2,000 rpm for 15 min and the supernatant combined with that from the first extraction. The pH was adjusted to 7.8 by the addition of concentrated ammonia solution. After standing for 2 hr the precipitate was removed by centrifugation at 2,000 rpm for 15 min. To each liter of supernatant was added 1.7 l of ice-cold ethanol and 2.8 l of ice-cold peroxide-free diethyl ether. After standing overnight at 4 C the precipitate

was collected by centrifugation at 2,000 rpm for 15 min. The precipitate was dried under a stream of nitrogen.

The ethanol-ether precipitate was then dissolved in 0.1M acetic acid, 2 g in 50 ml and dialysed in visking 18/32″ tubing against 0.1M acetic acid at 4 C for 48 hr. The retentate was chromatographed on a column of Sephadex G50 fine, 5 × 85 cm in 0.1M acetic acid and the effluent eluted between Kd 0.3 to 0.93 was then passed through a column of carboxymethyl cellulose, Whatman CM52, 2.5 × 15 cm, equilibrated in 0.1M acetic acid. The column was then washed with 200 ml 0.1M acetic acid, followed by 200 ml 0.3M acetic acid. A protein peak was then eluted by 0.2M sodium chloride in 0.3M acetic acid. This peak was freeze dried and then desalted using a 2.5 × 85 cm column of sephadex G10 in 0.1M acetic acid and freeze dried.

The IRP preparation was tested for insulin-releasing activity by intravenous injection into rats with glucose and demonstrating an increase in the 5-min plasma insulin level following injection compared with that following the injection of glucose alone, as described below under *in vivo* procedures.

The *UV* absorption characteristics of the material indicate a proteinaceous character and its molecular weight is probably of the order 3–4,000. Iso-electrofocusing shows a basic character (3).

Glucagon-like immunoreactive material (GLI) was assayed by Dr. S. R. Bloom of the Middlesex Hospital Medical School, London, and by Dr. K. D. Buchanan of the Queen's University, Belfast, using antisera raised against glucagon, but which cross-reacted equally well with enteric GLI. The highest estimate of GLI content was equivalent to 15 ng pancreatic glucagon per mg IRP. Batch G4 was tested by Dr. Viktor Mutt, of the Karolinska Institute, Stockholm and contained the equivalent of between 1 and 4 U secretin per mg IRP. No pancreozymin or cholecystokinin activity was detected. Batch A was assayed for secretin biological activity by Mr. V. D. Birkinshaw of Boots Pure Drug Company Ltd., Nottingham and found to contain the equivalent of 0.5 U secretin per mg IRP.

In vitro procedures

Rats were fasted overnight and killed by decapitation. The pancreas was removed, washed in ice-cold Krebs Ringer bicarbonate solution and dissected free of extraneous tissues in ice-cold incubation medium. Each pancreas was cut into 12 pieces, placed in a flask containing 6 ml Krebs Ringer Bicarbonate containing glucose, 3.3mM and incubated in a shaking water batch for 30 min at 37 C in an atmosphere of 95% oxygen, 5% carbon dioxide. The buffer incorporated Trasylol®, a proteinase inhibitor, 250 KIU/ml.

At the end of 30 min the pancreas pieces were transferred to new flasks containing 6 ml incubation medium at the glucose concentration required for the experiment and incubated for a further 15 min.

Twelve flasks were prepared, each containing 3 ml incubation medium with the appropriate glucose concentration. Six flasks contained no addition to the medium and served as controls. The other six contained the material under test for insulin-releasing activity. A piece of pancreas from four different rats was placed in each flask and the flasks incubated for 30 min. The pancreas pieces were weighed and the insulin content of the incubation medium measured by radioimmunossay. Insulin values were expressed as μU/ml insulin/mg pancreas/30 min.

The results of each set of 6 flasks were expressed as a percentage of the mean value for the 6 control flasks. The statistical significance of the values obtained was assessed by use of the Student's *t* test using Bessel's and Aspin-Welch corrections, except for cases in which the variance ratio required the use of the F-distribution.

In vivo procedures

Overnight-fasted Wistar rats weighing 300–350 g were used. Each rat was weighed and anaesthetized with sodium pentobarbitone (Nembutal®) 3.5–4.0 mg/100 g body wt. The Nembutal was diluted in saline to a concentration of 6 mg/ml and injected ip. Blood samples were obtained by cutting the tip of the tail and milking the tail vein.

Samples for glucose estimation were collected in 0.05 ml capillary pipettes and discharged into 0.95 ml 2% w/v perchloric acid.

Blood for insulin assay was collected into heparinized microcentrifuge tubes (Gelman-Hawkesley) and kept on ice until centrifuged. The centrifuge tubes were stored upright at

—15 C and the portion of the tube containing plasma cut off when required for use.

Glucose (0.625 g/kg body wt) was injected into the femoral vein as a 12.5% w/v solution over a period of 1½ min. IRP and other test substances were dissolved in 12.5% glucose solution for injection.

The amino acid mixture used for intravenous injection was a 10% w/v solution of hydrolysate (Aminosol®) Vitrunn Co., Sweden).

Glucose was measured by a glucose oxidase procedure using the Boehringer kit. Plasma insulin was measured using a double antibody radioimmunoassay procedure (6).

Results

Effect of IRP on rat pancreas pieces in vitro

The results and conditions of each test are listed in Table 1. IRP, 0.1 mg/ml was without effect on insulin release from pancreas pieces incubated at a glucose concentration of 3.3 mM, but significantly potentiated insulin release when incubated with 16.7 mM glucose. A smaller but significant stimulation of insulin release was seen when IRP was added to pancreas pieces incubated with 16.7 mM L-leucine.

Effect of secretin and pancreozymin on rat pancreas in vitro

Secretin and pancreozymin were used in combination in the rat pancreas piece preparation at a concentration of 1 U/ml of each. No significant effect on insulin release was observed in the presence of either 3.3 mM glucose or 16.7 mM glucose.

1. *Effect of IRP on amino acid induced insulin release* in vivo

Mean blood glucose and plasma insulin levels resulting from this experiment in two groups of eight animals are shown in Fig. 1. There was a greater rise in plasma insulin in rats given protein hydrolysate plus IRP than in those given protein hydrolysate alone but the difference was not significant ($p > 0.05 < 0.1$).

2. *Effect of secretin-pancreozymin in combination on response to intravenous glucose*

Mean blood glucose and plasma insulin values in two groups of eight rats given either glucose or glucose plus secretin and pancreozymin are shown in Fig. 2. Blood glucose levels differed significantly only at 30 min ($p < 0.05$), when they were lower in animals given secretin and pancreozymin in addition to glucose than in those given glucose alone. Peak plasma insulin levels at 5 min were significantly higher ($p < 0.001$) in animals receiving secretin and pancreozymin (238 ± 16.3 μU/ml) than in those receiving glucose alone (136 ± 7.9 μU/ml). Differences at other times were not significant.

TABLE 1.

Incubation conditions	No. of flasks	Insulin release as % control	p value difference from control
Glucose 3.3 mM	12	100 ± 10.723	
Glucose 3.3 mM plus IRP 0.1 mg/ml	12	118.66 ± 16.91	>0.15
Glucose 16.7 mM	12	100 ± 14.664	*
Glucose 16.7 mM plus IRP 0.1 mg/ml	12	287.07 ± 66.337	<0.01
Glucose 3.3 mM, Leucine, 16.7 mM	6	100 ± 13.253	
Glucose 3.3 mM, Leucine, 16.7 mM plus IRP 0.1 mg/ml	6	146.66 ± 17.255	<0.025
Glucose 3.3 mM	6	100 ± 15.13	
Glucose 3.3 mM plus secretin and pancreozymin 1.0 U/ml	6	68.67 ± 13.30	p > 0.05
Glucose 16.7 mM	6	100 ± 16.78	
Glucose 16.7 mM plus secretin and pancreozymin 1 U/ml	6	126.39 ± 18.08	p > 0.15

* By F distribution.

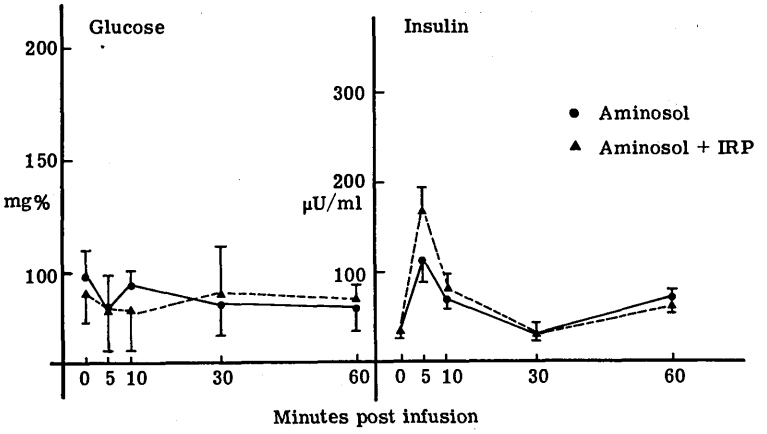

FIG. 1. Effect of IRP (0.5 mg/kg) on plasma insulin and blood glucose response to intravenous amino acids. A 10% w/v solution of casein hydrolysate (Aminosol®) was given, 5 ml/kg body weight. IRP (batch G4) was dissolved to give a concentration of 0.1 mg/ml. ——●—— mean ± alone; ---▲--- mean ± SEM in 8 rats given aminosol plus IRP.

3. *Effect of secretin plus pancreozymin administered with intravenous saline solution*

Mean blood glucose and plasma insulin levels in six rats given saline alone iv and rats given saline plus secretin and pancreozymin are shown in Fig. 3. There was no significant difference between glucose and insulin levels in the two groups.

4. *Dose response to IRP*

The curve obtained by plotting glucose dependent insulin releasing activity as a percentage of control values against dose is shown in Fig. 4. These data were obtained by measuring plasma insulin values 5 min after the administration of glucose or glucose plus IRP. Significant augmentation of insulin release ($p < 0.01$) was demonstrated by the smallest dose level used in this experiment, 62.5 μg IRP/kg body wt. Increasing the dose increased the response and there was a tendency to saturation. A plot of glucose dependent insulin-releasing activity against log dose is shown in Fig. 5.

5. *Dose response to secretin*

The plasma insulin response at 5 min to the addition of four doses of secretin to glucose injections under the same conditions as used to test the dose response to IRP are shown in Fig. 4. At no dose was there a significant insulin-reaching effect.

Discussion

The active component of the partially purified IRP preparation used in the present studies possesses the property of potentiating glucose-stimulated insulin release in the rat. It has no effect on insulin release at basal blood glucose concentrations.

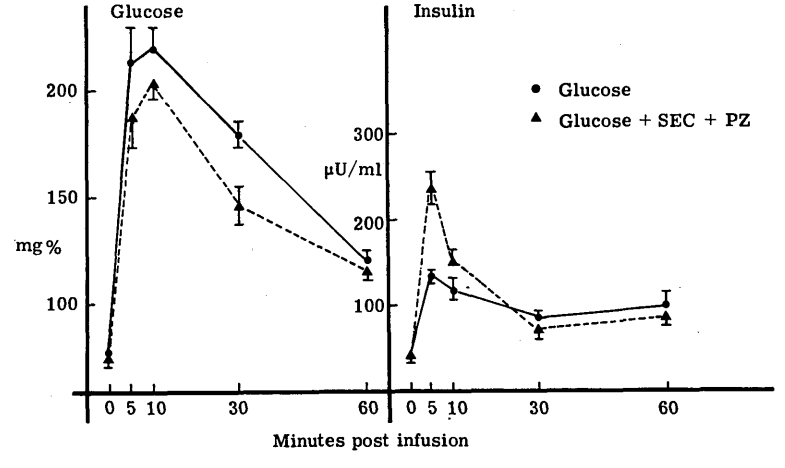

FIG. 2. Effect of secretin (0.5 U/kg) plus pancreozymin (0.5 U/kg) on the plasma insulin and blood glucose response to intravenous glucose. 12.5% w/v glucose solution was given, 5 ml/kg body wt. Secretin and pancreozymin were dissolved in the glucose solution to give a concentration of 0.1 U/ml of each hormone. ——●—— mean ± SEM in 8 rats given glucose alone; ---▲--- mean ± SEM in 8 rats given glucose plus secretin and pancreozymin.

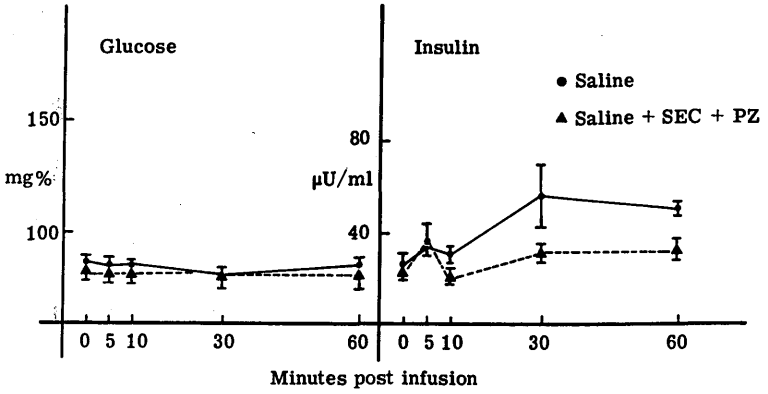

FIG. 3. Effect of secretin (0.5 U/kg) plus pancreozymin (0.5 U/kg) at basal blood glucose concentrations. 0.9% saline was given intravenously, 5 ml/kg body wt. Secretin and pancreozymin were dissolved in saline to give a concentration of 0.1 U/ml of each hormone. ——●—— mean ± SEM in 8 rats receiving saline alone; ---▲--- mean ± SEM in 8 rats given saline plus secretin and pancreozymin.

Similar effects are observed both *in vitro* and *in vivo*. Thus, the behavior of IRP with respect to glucose-stimulated insulin release in the rat corresponds to that postulated (2) for an hypothetical intestinal hormone designated "Incretin" but now referred to as "Glucose-dependent insulin-releasing hormone (GIRH)"—which would be responsible for the greater insulin secretory response to oral as compared to intravenous glucose (1). These findings are in agreement with earlier reports (2,7) showing potentiation of glucose-stimulated insulin release *in vitro* by extracts prepared from porcine intestine.

A less marked augmentation of amino acid-stimulated insulin release is shown by IRP *in vitro* and *in vivo*. The fact that this response is much smaller than that produced in the presence of hyperglycaemia may indicate that IRP does not exert a significant effect on amino acid-stimulated insulin release under physiological conditions. A stronger candidate for that role would be pancreozymin (2,8).

The results of dose-response experiments show a curve typical of many biologically active agents. The lowest dose of IRP used in the present series of experiments was 62.5 μg IRP/kg body wt. This was sufficient to produce a significant effect on insulin release. Since the IRP preparation used in these studies was relatively crude it is probable that much smaller doses of pure IRP would evoke a significant insulinemic response.

The log-linear dose response to IRP ob-

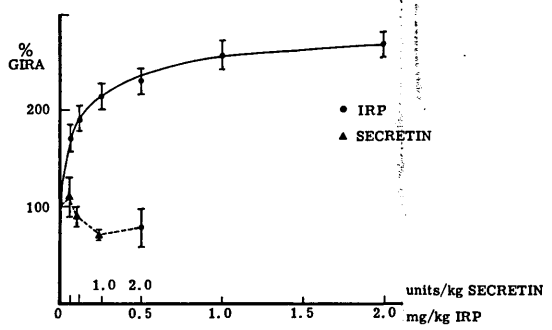

FIG. 4. Dose-response to IRP and secretin in rats given intravenous glucose. Each animal received 5 ml/kg body wt of a 12.5% w/w glucose solution containing IRP or secretin at the dose indicated. Each point is the mean ± SEM of the plasma insulin level at 5 min after the injection for 8 rats. ——●—— IRP; ---▲--- Secretin; % GIRA represents glucose dependent insulin-releasing activity as a percentage of the mean control value for glucose alone.

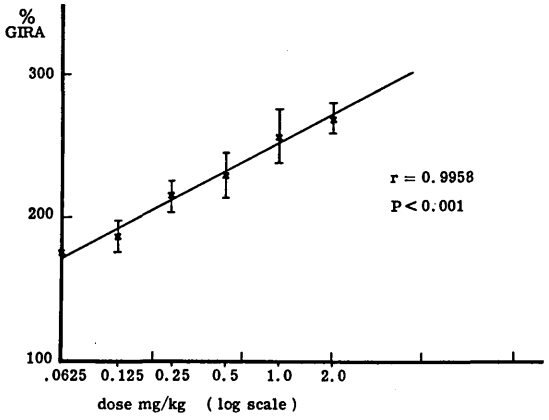

FIG. 5. Dose-response to IRP. The same data for dose-response to IRP as shown in Fig. 4 are plotted against log-dose of IRP.

tained *in vivo* may provide the basis of a quantitative bioassay for IRP activity.

The action of secretin and pancreozymin in combination was similar to that of IRP, with two important exceptions. First, IRP at a dose of 0.5 mg/kg body wt produced a marked reactive hypoglycaemia after 45 min, whereas secretin plus pancreozymin did not. Secondly, the peak mean plasma insulin level produced by glucose plus secretin and pancreozymin was lower than that produced by glucose and IRP, 0.5 mg/kg body weight. This experiment was performed to examine the possibility that the insulin-releasing activity of IRP might be accounted for by the combined action of secretin and pancreozymin contaminants. This would seem to be most unlikely, however, in view of the low content of secretin biological activity of IRP and the absence of pancreozymin or cholecystokinin activity. Secretin alone in quantities up to and including the maximum level of secretin contamination of IRP had no significant effect on 5-min plasma insulin levels *in vivo*. The failure of secretin and pancreozymin to stimulate insulin release *in vitro* is consistent with earlier observations made with the two hormones separately (2,9,10). Other studies (11,12,13) have reported secretin and pancreozymin stimulation of insulin release *in vitro*, but their effect does not appear to be related to glucose concentration. It seems exceedingly unlikely that secretin and pancreozymin either separately, or in combination, are responsible for the insulin-releasing activity of IRP.

There is increasing evidence (9,14,15) that gut GLI does not possess insulin-releasing activity. It seems unlikely, therefore, that the small content of GLI in the IRP preparations contributed to its insulin-releasing activity.

Gastric Inhibitory Polypeptide (GIP) recently purified from porcine duodenum (16) has been found to augment glucose-estimated insulin release. It is not yet possible to relate the activity of this peptide to the findings discussed here.

The degree to which IRP potentiates glucose-stimulated insulin release is sufficient to account for the greater insulin-releasing potency of oral as compared to intravenous glucose. It is likely, therefore, that the active component of IRP is the hormone proposed for the intestinal regulation of glucose-stimulated insulin release.

Acknowledgments

This work was supported by generous grants from the British Diabetic Association and the Wellcome Foundation.

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FIBROSARCOMAS INDUCED BY COBALT CHLORIDE ($CoCl_2$) IN RATS

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LABORATORY ANIMAL - 1976 - in press

SUMMARY

In a study of the long term effects of cobalt chloride ($CoCl_2$) in the rat hyperlipaemia persisted for more than 12 months.

Nine out of 20 cobalt chloride treated rats and one control rat died within the first year and eight out of the surviving animals developed fibrosarcomas. In four of the animals the tumour was far removed from the injection sites.

The results are discussed with reference to the use of cobalt chloride treatment for anaemia in patients with chronic renal failure.

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cultured islets and the stimulatory action seemed to be enhanced as the glucose concentration of the culture medium increased. – It is concluded that exposure of isolated pancratic islets for one week to a high glucose concentration causes an exaggerated glucagon response to both arginine and glucose. It remains unclear to what extent this is due to the high insulin concentration present in the culture system.

250. Glucose Effects on Calcium Fluxes in Pancreatic Islets, Using Lanthanum to Distinguish between Superficial and Intracellular Pools.

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Fluexes of $^{45}Ca^{2+}$ were studied in islets from *ob/ob*-mice. La^{3+} blocked both influx and efflux of $^{45}Ca^{2+}$; in studies of intracellular $^{45}Ca^{2+}$ the islets were washed with 2 mM La^{3+} for 1 hour after incubation. The uptake of $^{45}Ca^{2+}$ in islets ixposed to 3 mM D-glucose reached an apparent equilibrium after 2 hours at which time the intracellular concentration of exchangable Ca^{2+} was about 7 mmol/kg dry weight (with 2.6 mM Ca^{2+} in medium). Raising the D-glucose concentration to 20 mM enhanced the $^{45}Ca^{2+}$ uptake whether or not the islets had first been equilibrated with the isotope. The effect could not be reproduced with equimolar L-glucose. – The rate of release of intracellular $^{45}Ca^{2+}$ was the same whether the islets had been preloaded with 3 or 20 mM D-glucose; halftime for release was 30 min. Thus, $^{45}Ca^{2+}$ that had been taken up in response to 20 mM D-glucose appeared to be released much more slowly than the bulk of intracellular $^{45}Ca^{2+}$. – It is concluded that 20 mM D-glucose caused a net uptake of Ca^{2+} into the β-cells. This uptake was probably not regulated at the level of the plasma membrane but more likely reflected an increased affinity of some intracellular compartment for the ion.

251. A Computer Evaluation System for the Study of the Natural History of Diabetes.

H.K. Selbmann, W. Kreutzfeld, K. Schöffling, K. Überla. Univ. München, Inst. f. med. Informationsverarb., Statistik, Univ. Göttingen, Med. Klinik und Poliklinik, Univ. Frankfurt, Zentrum der Inneren Medizin, Abt. Endokrinologie, FRG.

A retrospective study of 1302 diabetics was conducted in the outpatient departments of the Universities of Frankfurt and Göttingen. The patients were observed every year, over a period starting usually with the year of diabetes recognition, and ending with the closing date of the study in 1971. So approximately 11,000 years of diabetic life directly observed are available for statistical analyses. An efficient evaluation of such large volumes of data can only be done by computer information systems. The presented system SAVOD-L allows the statistical evaluation of the data in a dialogue with the computer. The immediate answering of an open list of questions in form of frequency information, histogrammes, crossclassification tables or survival curves enables an intensive exploitation of the data. – The features of the information system are demonstrated on a display terminal located in the congress area. This terminal is connected with the computer of the medical faculty of the University of Munich. So the participants of the congress have the opportunity to ask questions directly of the information system.

252. Elevations of Sorbitol and Myoinositol in Diabetic and Nondiabetic Patients Who Have Impaired Renal Function.

C. Servo, E. Pitkänen. IV Department of Internal Medicine, University of Helsinki, SF-00170 Helsinki, 17, Finland.

We studied the mechanisms responsible for elevations in concentrations of sorbitol in diabetes and myoinositol in uremia. – Concentrations of polyols were determined by gas-liquid chromatography in plasma, red cells or cerebrospinal fluid (CSF), or in all three; 10 diabetic patients with nephropathy, 10 non-diabetic uremic patients and 14 non-diabetic patients on dialysis were studied. Levels of sorbitol were significantly elevated (p 0.01) in CSF of diabetic patients and in red cells of both diabetic and dialyzed patients, an observation not previously reported. During dialysis red cell sorbitol levels rose by 20%. – Concentrations of myoinositol, normally elevated in CSF, were highest in plasma of almost all patients, a finding which in uremic patients correlated negatively with creatinine clearance. During dialysis, plasma levels fell by 45–80% and red cell levels by 0–15%. – Our results show that difference in osmolar gradients between red cell and plasma polyols were too small to cause intracellular damage and that elevations in levels of CSF-sorbitol were caused by synthesis in situ and not by the uremic state. Elevations of myoinositol in uremia were likely to result from tissue retention and impaired degradation in the kidneys and seem therefore to be an index of kidney damage.

253. The Role of Pancreas in Hyperlipaemic Rats.

A. A. Shabaan, V. Marks. Department of Biochemistry, University of Surrey, Guildord, Surrey. GU2 5XH., U. K.

Experimental hyperlipaemia produced in the rat by cobalt chloride ($CoCl_2$) injection can serve as a model for studying the mechanism and the factors involved in human hyperlipaemia. – Rats were made hyerlipaemic by subcutaneous injection of $CoCl_2$. Tissue distribution of cobalt was studied by injection of radioactive cobalt (^{57}Co). Hepatocytes from cobalt chloride treated and control rats were studied and compared in monolayer tissue cultures. – The pancreas contained more cobalt than any other tissue apart from the liver and kidneys and the islets showed evidence of histological damage. Glucose uptake by hepatocytes isolated from the $CoCl_2$ treated rats was unaffected by addition of either insulin or glucagon in contrast to hepatocytes isolated from untreated controls. – $CoCl_2$ induced hyperlipaemia is associated with abnormal control of carbohydrate metabolism in the liver, possibly secondary to islet cell damage.

254. Effect of Insulin on Activity of Lipogenesis Enzymes and Growth of Cultured Human Fibroblasts.

E. Shafrir, E. L. Bierman. Department of Medicine, University of Washington School of Medicine, Seattle, Washington, USA.

Since little is known about the regulation of lipogenesis in human tissues, the capactiy of fatty acid synthesis in human skin fibroblasts has been assessed. The fibroblasts, obtained from normal adult donors, were grown to confluency in a modified Dulbecco's medium. The medium was then replaced with a fresh one containing 10% fetal calf serum made lipoprotein free by ultracentrifugation at d = 1.25 and free fatty acid free by charcoal absorption. From 20 μU/ml to 20,000 μU/ml of purified single component insulin (Lilly) were added and the cells incubated for 3 days. Proliferation of cells was discernible even at the lowest insulin concentration and a linear relationship between log of insulin concentration and cell growth was apparent. In the presence of 2,000 μU/ml growth was increased by 28 to 45% with various cell strains. – The activity of the rate-limiting enzyme of fatty acid synthesis, acetyl-CoA carboxylase was determined in 100,000 g supernatant fraction, by fixation of $^{14}CO_2$ after maximal activation with citrate. The activity ranged from 0.15 to 0.80 nmol · min^{-1} · mg^{-1} of soluble protein in various cell strains compared to 1 and 24 nmol · min^{-1} · mg^{-1} in human subcutaneous and rat epididymal adipose tissues, respectively. Increasing amounts of insulin induced a gradual rise in enzyme activity independent of the increase in cell number. At 2,000 μU/ml for 3 days, the activity rose 1.5 to 4-fold when expressed per cell, or per protein or DNA content. Likewise, insulin induced increases in the activity of other regulatory enzymes of lipogenesis, ATP-citrate lyase, NADP-malate dehydrogenase and fatty acid synthetase. The activity of the enzymes and the extent of induction tended to decrease after multiple passages of cells in culture. The presence of fatty acid synthetic enzymes and their amenability to insulin induction, in a fashion similar to that in liver and adipose tissue of experimental animals, render the cultured fibroblasts a convenient model living system for the study of regulation of lipogenesis in man.

255. A Possible Role of Glucagon in the Mechanism of Somatostatin – Induced Inhibition of Insulin Release.

J. Sieradzki, H. Schatz, C. Nierle and E.F. Pfeiffer. Dept. of Internal Medicine, Endocrinology and Metabolism, University of Ulm, FRG. Alexander von Humboldt-Fellow 1974/75. Supported by Deutsche Forschungsgemeinschaft, SFB 87 Endokrinologie.

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Sustained Insulin Release in Response to Intravenous Infusion of Insulin Releasing Polypeptide (IRP) in the Rat

Ahmad A. Shabaan, M.Sc., Desmond S. Turner, Ph.D., and Vincent Marks, D.M., Surrey, England

SUMMARY

A greatly elevated and sustained insulinemic response was evoked in rats by the intravenous infusion of a solution of IRP in glucose compared with that produced by glucose alone. Larger doses of IRP produced correspondingly greater increases in insulin release.

IRP enhanced glucose disposal up to a finite limit despite higher plasma insulin levels suggesting that there is a maximum rate of glucose disposal under conditions of intense insulin stimulation. DIABETES *23*:**902-04, November, 1974.**

Intestinal insulin releasing polypeptide (IRP), a crude extract of porcine intestine of molecular weight between 3,000-5,000 is known to be free from contamination by most of the well recognized gastrointestinal hormones including secretin, cholecystokinin-pancreozymin (PZ-CCK) and enteroglucagon.[1] The possibility of contamination by gastrin inhibitory polypeptide (GIP) has been considered,[2] but has not been resolved.

IRP has been shown to enhance insulin release in response to intravenous glucose in the rat[3] and the baboon.[4] It has been suggested that IRP may contain the intestinal hormone responsible for the greater insulinemic response to oral as compared to intravenous glucose.[5] None of the well characterized intestinal hormones satisfactorily explains all of the observed phenomena.

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Accepted for publication August 16, 1974.

Information on the biological activity of IRP published to date has largely been concerned with the insulinemic response to its rapid intravenous injection. However, since the insulin secretory response to oral glucose is maintained over relatively prolonged periods IRP must be shown, if it is to qualify as the intestinal mediator of augmented insulin release, to be capable of exerting a sustained effect on insulin release when given by constant intravenous infusion. This paper describes experiments performed in rats to test this proposition.

MATERIALS AND METHODS

Overnight fasted Wistar rats, aged twelve weeks and weighing 300-325 gm., were anesthetized with Nembutal (40 mg. per kg. body weight) intraperitoneally. IRP, prepared by the method of Turner et al.,[1] was dissolved in glucose solutions (7 per cent or 14 per cent w/v) and given by constant intravenous infusion into a femoral vein for thirty minutes at a rate of 0.1 ml. per minute.

Controls received glucose infusions without added IRP at the same concentrations as the test animals (i.e. 25 mg. per kg. body weight per minute, or 50 mg. per kg. body weight per minute). The detailed protocol of each experiment is given in the Results section.

Blood was sampled from the tail vein at frequent intervals. Blood glucose was measured using an automated glucose-oxidase method and plasma insulin by a double antibody radioimmunoassay.[6]

RESULTS

1. *Effect of different doses of IRP on plasma insulin and blood glucose*

The mean blood glucose and plasma insulin levels observed in each of three groups of four rats given intravenous infusions of 14 per cent w/v glucose plus IRP, or glucose alone for the control group, are shown in figure 1.

Plasma glucose levels were not significantly different at five or ten minutes after starting the infusion, but at twenty minutes rats receiving glucose alone had significantly higher ($p < 0.05$) blood glucose levels than those receiving glucose plus IRP. This continued for the rest of the experiment. Blood glucose levels in the two groups of rats receiving different doses of IRP were similar throughout.

Within five minutes, and at both (33 and 107 μg/kg. per minute) dose levels, IRP caused a significant ($p < 0.01$) and sustained hyperinsulinemic response compared with that produced by glucose alone. With the larger doses of IRP plasma insulin concentration averaged over 2,000 μU/ml. and were the highest sustained levels ever observed in this laboratory. In all cases plasma insulin levels fell precipitously at the conclusion of the intravenous infusion and did not differ significantly from each other at either forty-five or sixty minutes.

These results show that both doses of IRP depressed the blood glucose concentration to the same extent when compared with the glycemia produced by glucose alone.

2. *Effect on plasma insulin and blood glucose of a constant dose of IRP with different glucose loads*

Figure 2 illustrates mean blood glucose and plasma insulin levels in each of four groups of four rats thirty minutes after commencing an intravenous infusion of glucose (either 25 or 50 mg. per kg. per minute) with or without IRP (33 μg/kg. per minute). Mean blood glucose levels were higher in the rats that received the

Blood glucose responses to infusions of IRP at different dose rates during continuous glucose infusion

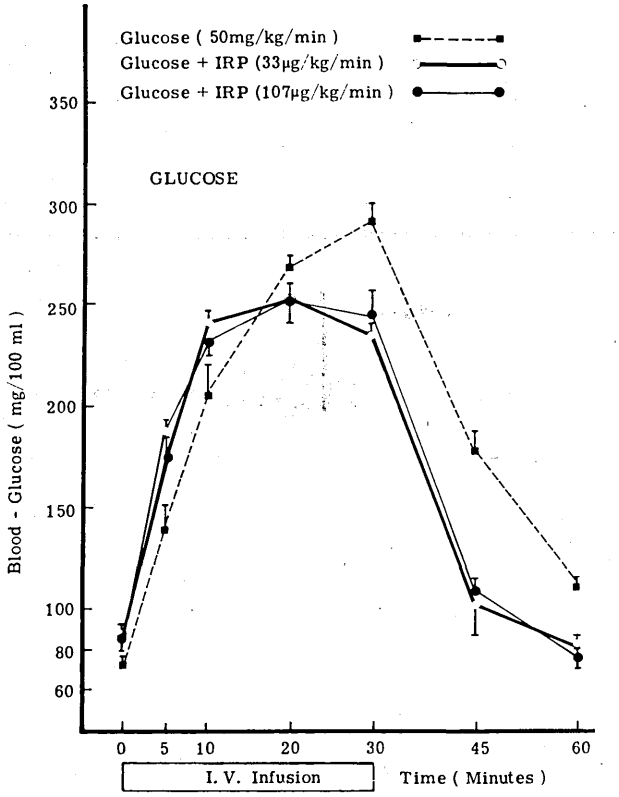

FIG. 1. Mean blood glucose levels (± S.E.M.) in three groups of four rats given a thirty minute intravenous infusion of:
1. glucose alone (50 mg. per kg. per minute)
2. glucose (50 mg. per kg. per minute) plus IRP (33 μg/kg. per minute)
3. glucose (50 mg. per kg. per minute) plus IRP (107 μg/kg. per minute)

Plasma insulin responses to infusions of IRP at different dose rates during continuous glucose infusion

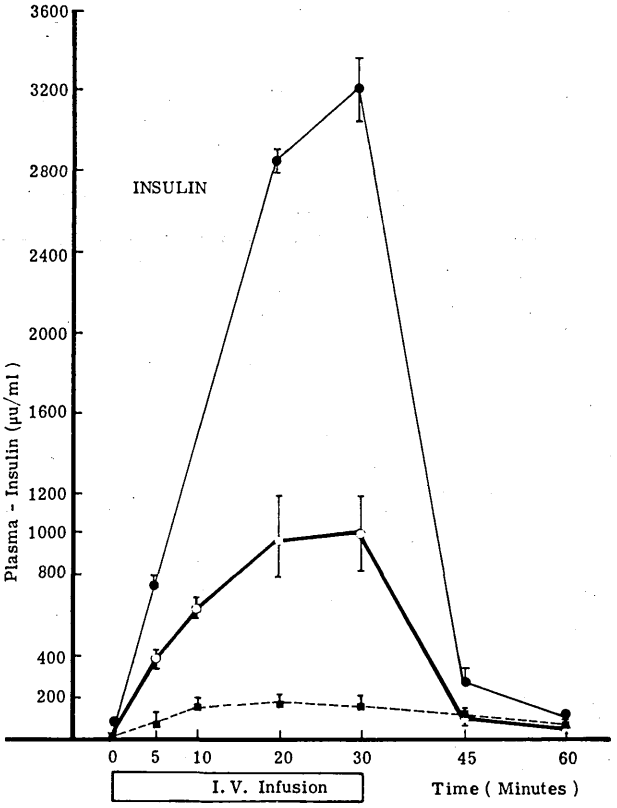

FIG. 1A. Mean plasma insulin levels (± S.E.M.) in three groups of four rats given a thirty minute intravenous infusion of:
1. glucose alone (50 mg. per kg. per minute)
2. glucose (50 mg. per kg. per minute) plus IRP (33 μg/kg. per minute)
3. glucose (50 mg. per kg. per minute) plus IRP (107 μg/kg. per minute)

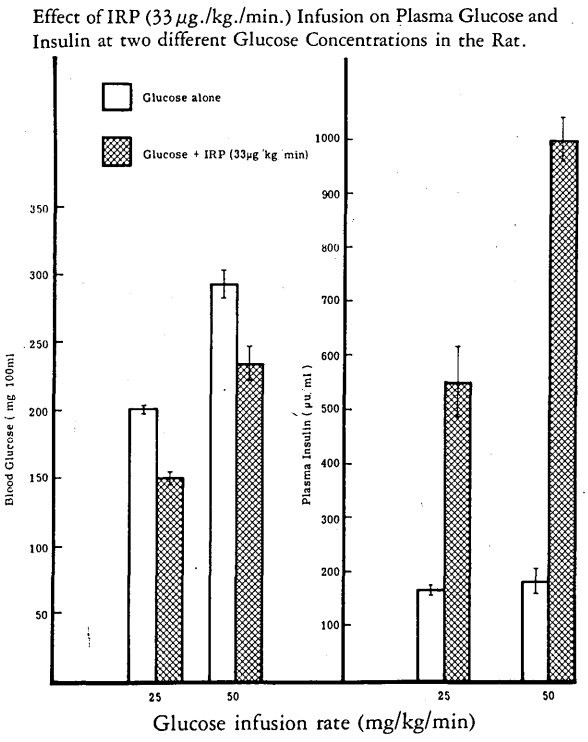

FIG. 2. Mean (± S.E.M.) blood glucose (on the left) and plasma insulin levels (on the right) in two groups of four rats after thirty minutes intravenous infusion containing IRP at constant dosage (33 μg/kg. per minute) and different amounts of glucose (25 and 50 mg. per kg. per minute). Two control groups, each of four rats, were infused with glucose alone at the appropriate rate.

larger glucose load, but in each case were significantly lower in the animals that also received IRP. Plasma insulin levels were higher in animals receiving the larger dose of glucose and the augmentory effect of IRP on insulin release was significantly greater ($p < 0.01$).

DISCUSSION

The experiments described indicate that IRP given by constant intravenous infusion is capable, in the presence of mild to moderate hyperglycemia, of causing a marked and sustained augmentation of insulin secretion which is of more than sufficient magnitude to explain the greater insulin releasing potential of oral versus intravenous glucose.[5] Indeed, the extent of the hyperinsulinemia observed with large doses of IRP indicates its enormous insulinotropic potency even though the preparation currently available is far from pure.

The greater plasma insulin response observed with the larger doses of IRP, under these conditions, is consistent with the dose response relationship between IRP and insulinemia, previously reported.[1] Even so both doses of IRP had the same effect on glucose tolerance judging from the magnitude of the hyperglycemic response to the intravenous glucose load and the rate of glucose disappearance from the blood ($\frac{t}{2}$) at the conclusion of the infusion. These observations suggest that, in vivo, there is a maximum rate of glucose disposal and further increments above a still poorly defined plasma insulin concentration have no additional effect upon glucose tolerance.

ACKNOWLEDGMENT

Thanks are due to the British Diabetic Association and Wellcome Foundation, Beckenham, for generous support.

The present study incorporates work accepted for the award of M.Sc. in Clinical Biochemistry to Ahmad A. Shabaan at the University of Surrey.

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The Effect of Adrenaline on Insulin Releasing Polypeptide (IRP) Mediated Insulin Release *in vivo* in the Rat*

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Summary. Intestinal insulin releasing polypeptide (IRP) has been shown to potentiate the release of insulin in response to glucose both *in vitro* and *in vivo* in the rat. Adrenaline reduced the insulin secretory response to the intravenous infusion (I.V.) of glucose and to the infusion of glucose with IRP given either as a rapid injection or as constant infusion.

Key words: Adrenaline, IRP, intestinal hormones, insulin release, glucose infusion, secretin, glucose tolerance.

The nature of the mediator of intestinal augmentation of insulin secretion has aroused much speculation since the phenomenon was first described by McIntyre *et al* in 1964 [1]. Most of the well-known and characterised intestinal hormones have been proposed at some time or other but none satisfactorily fulfils the role of the intestinal mediator [2, 3]. An intestinal insulin releasing polypeptide [IRP] which contains none of these hormones has recently been isolated and partially purified [4, 5]. It has been shown to potentiate the release of insulin in response to glucose, both *in vitro* and *in vivo*, but to have no, or little effect in the absence of induced hyperglycaemia [5, 6].

* The present study incorporates work accepted for the award of M.Sc. in Clinical Biochemistry to Ahmad A. Shabaan at the University of Surrey.

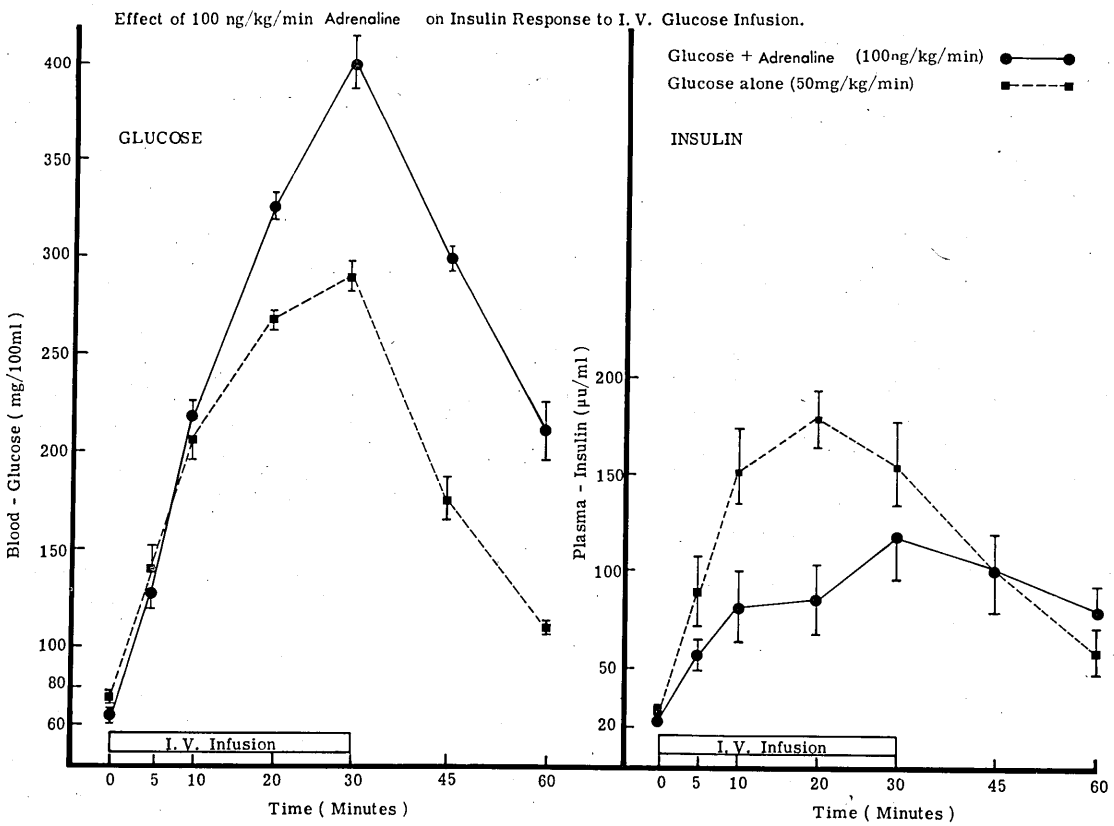

Fig. 1. Mean blood glucose and plasma insulin levels (± S.E.M.) in two groups of six rats given either an I.V. infusion of glucose (50 mg/kg/min) or glucose plus adrenaline (100 ng/kg/min)

The inhibitory effect of adrenaline and nor-adrenaline upon pancreatic insulin release provoked by intravenous glucose is well recognised [7, 8]. Much less information is available about their effect upon oral glucose mediated insulin secretion. In a brief note Langs and Friedberg [9] reported that, in man, the I.V. infusion of adrenaline at a rate sufficient completely to suppress glucose stimulated insulin secretion, did not suppress the effect of oral glucose on insulin release. This was not confirmed [10]. Nelson *et al* [11] reported that adrenaline did not suppress secretin induced insulin release and on this basis suggested that in man secretin might be a suitable candidate for the hypothetical alimentary mediator.

In the present paper the results of studies in the rat concerning the effect of adrenaline on the insulin response to the intravenous infusion of glucose and of glucose plus IRP are reported and their possible relevance to the nature of the intestinal mediator discussed.

Materials and Methods

Overnight fasted Wistar rats aged 12 weeks, weighing 300—325 g, were anaesthetised with Nembutal® (40 mg/kg body weight, I.P.). IRP, prepared as previously described [5] was dissolved in saline or, together with adrenaline (B.P. MacCarthys), either in 7% or 14% w/v glucose solution, containing ascorbic acid (2 mg/ml) as adrenaline preservative. The constant infusion of approximately 0.1 ml/min for 30 min of the glucose or glucose plus adrenaline solutions was given into a femoral vein using a syringe pump. The exact

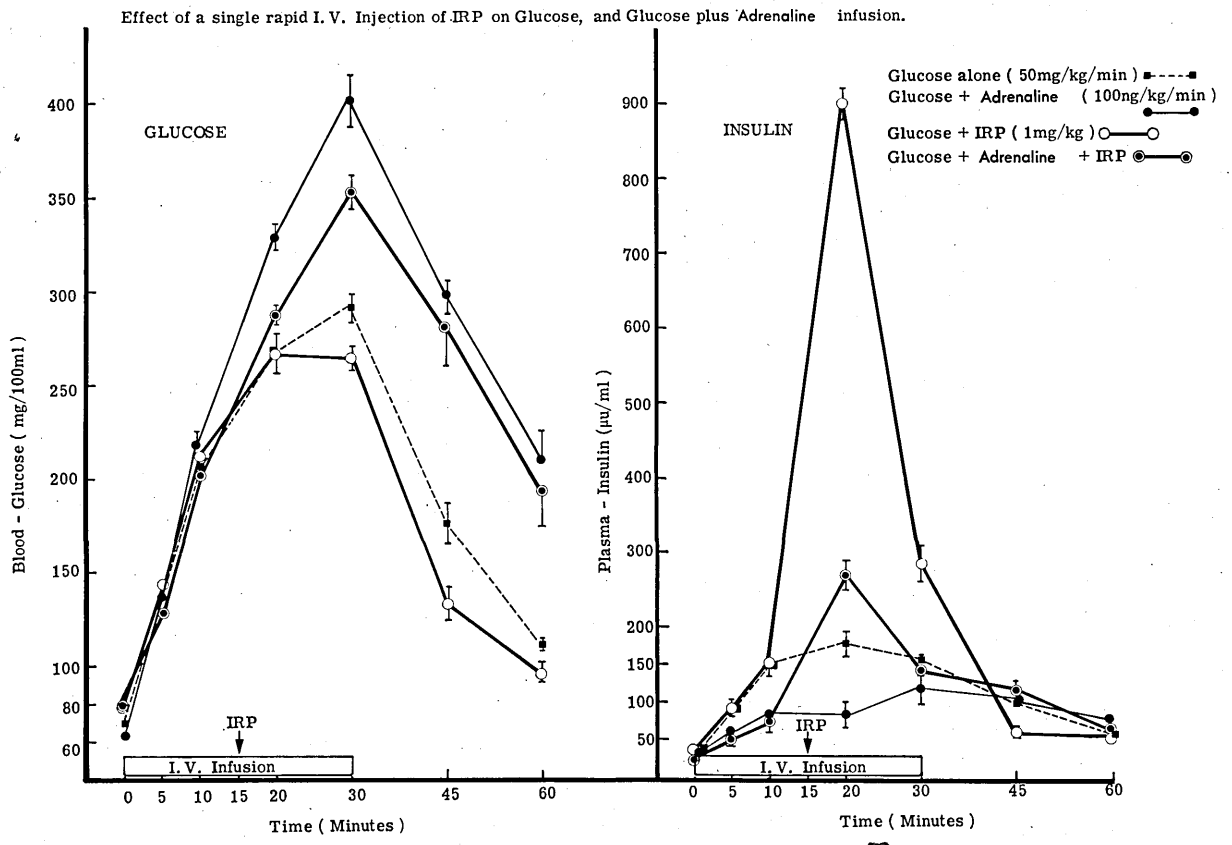

Fig. 2. Mean blood glucose and plasma insulin levels (± S.E.M.) in four groups of Wistar rats given an intravenous glucose infusion (50 mg/kg/min) over 30 min

Group No.	No. of Rats	Glucose (50 mg/kg/min)	IRP (in saline) (1 mg/kg)	Adrenaline (100 ng/kg/min)
1	6	+	—	—
2	6	+	—	+
3	4	+	+	—
4	4	+	+	+

protocol followed in each experiment is given in the results section.

Blood for glucose and plasma insulin measurements was obtained at regular intervals from the tail vein. Blood glucose was measured on the Autoanalyzer (Technicon Ltd) using an automated glucose oxidase method (Boehringer). Plasma insulin was determined by a double antibody radioimmunoassay procedure [12] using human insulin as standard.

The effect of adrenaline on insulin release after a rapid injection of IRP dissolved in saline was studied 15 min after the start of an infusion of either glucose or glucose plus adrenaline. The results, shown in Fig. 2, indicate that insulin release following rapid intravenous injection of IRP was markedly, but not completely, inhibited by adrenaline ($p<0.001$). The effect of adrenaline on insulin release provoked by a continous infusion of IRP is shown in Fig. 3. In this experiment glucose was infused at a lower rate than in previous experiments, which accounts for the lower blood glucose levels throughout. The marked and sustained elevation of plasma insulin produced by IRP plus glucose, compared with that produced by glucose alone, was significantly attenuated, but not abolished, by adrenaline at all time intervals during the infusions ($p<0.001$ at 20 min).

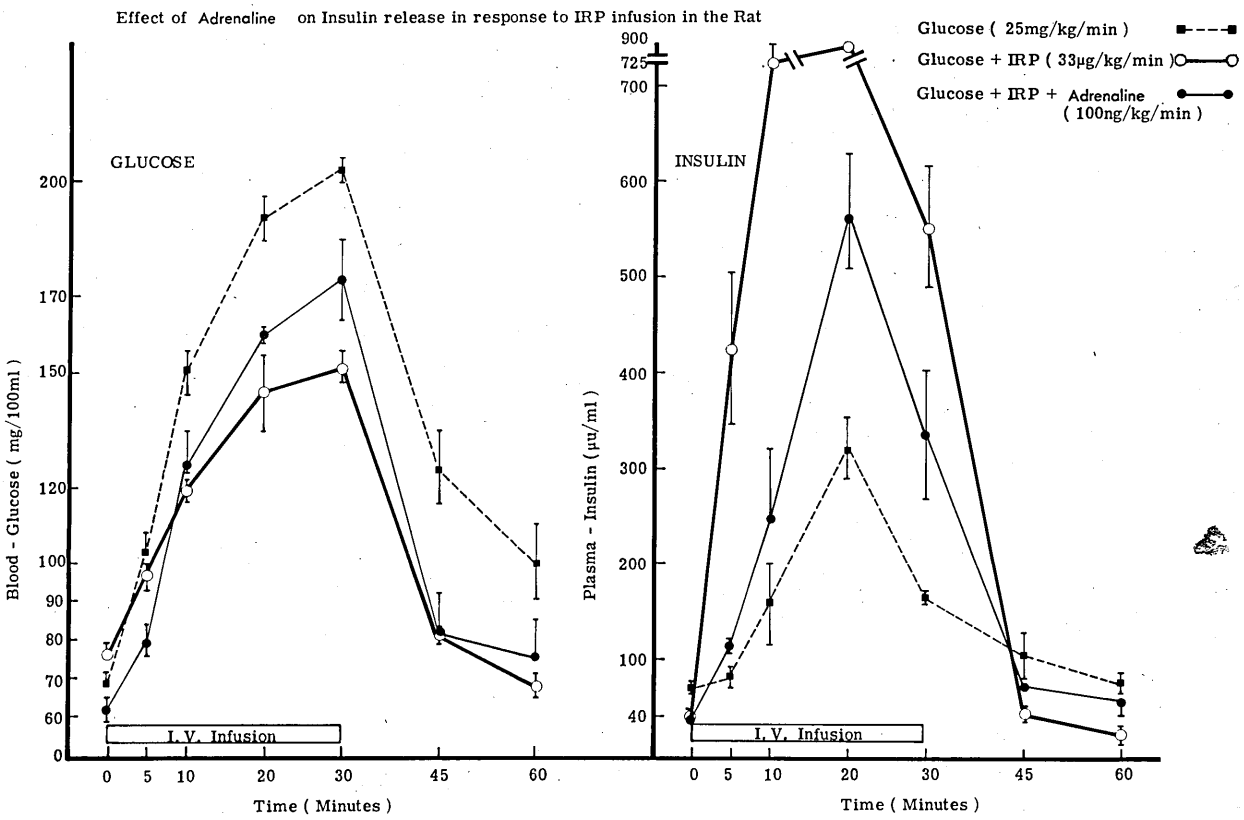

Fig. 3. Mean blood glucose and plasma insulin levels (± S.E.M.) in three groups of four rats. The first (control) group was given an I.V. glucose infusion (25 mg/kg/min) for 30 min and the second was given glucose plus adrenaline (100 ng/kg/min). The third group received IRP (33 µg/kg/min) in addition to glucose and adrenaline

Results

Preliminary experiments were carried out to find the smallest dose of adrenaline to suppress insulin secretion in response to I.V. glucose infusions in the rat. These experiments showed that adrenaline in doses within the range 10—60 ng/kg/min, which had previously been reported as effective in man, [8] did not cause significant inhibition of insulin release in the rat. Adrenaline at a dose level of 100 ng/kg/min inhibited insulin release at all time intervals (Fig. 1), despite blood glucose levels much higher than in animals receiving glucose alone. This dose of adrenaline was used in all subsequent experiments.

Discussion

The dose of adrenaline found in the present study to be effective as an inhibitor of insulin secretion in re-

sponse to intravenous (I.V.) glucose infusions in rats was larger than that reported by Cerasi *et al.* [8] to be effective in humans, but similar to that actually used in man by most previous investigators [7, 9, 10, 12, 13].

It is clear from the experiments described that adrenaline markedly inhibits the potentiating effect of IRP upon glucose-stimulated insulin secretion in the rat. Whether this is evidence for or against its merits as a candidate for the role of alimentary mediator of insulin release is uncertain. In contrast to the plethora of studies demonstrating inhibition by catecholamines of glucose mediated insulin release *in vivo* and *in vitro*, both in animals and in man, the only studies of their effect upon alimentary mediated insulin release known to us are those of Langs and Friedberg [9] and Fallucca *et al.* [10]. The latter showed, in three post-gastrectomy patients, a substantial and highly significant, though incomplete, inhibition by adrenaline of the exaggerated insulinaemic response to oral versus intravenous glucose.

IRP would seem, therefore, to qualify for further consideration as an intestinal mediator of glucose stimulated hyperinsulinaemia.

Acknowledgements. Thanks are due to the British Diabetic Association and Wellcome Foundation, Beckenham, for generous support.

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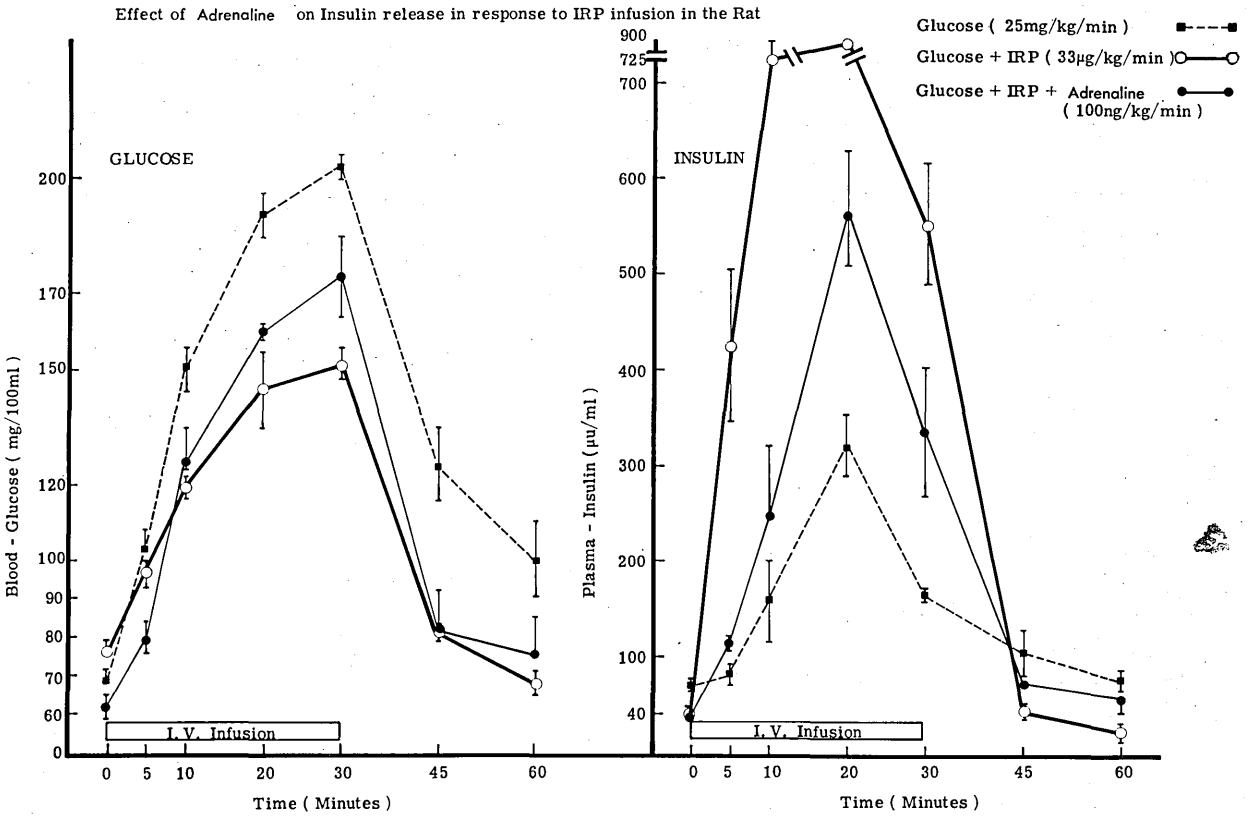

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