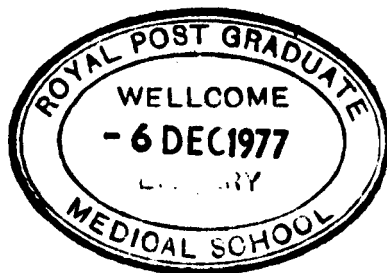


~~CLINICAL AND~~ EPIDEMIOLOGICAL STUDIES
OF THE CONCENTRATIONS OF INSULIN IN MAN

By

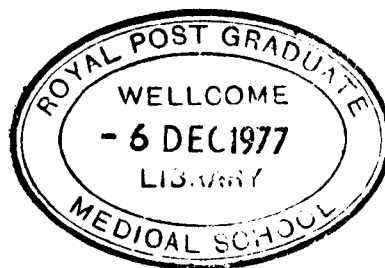
Timothy A. Welborn



A thesis
presented for the degree of
Doctor of Philosophy
at the University of London

BEST COPY AVAILABLE.

VARIABLE PRINT QUALITY



ABSTRACT

Clinical and epidemiological studies were conducted to identify factors related to the wide range of serum-insulin values encountered in man. A reliable modification to the double antibody immunoassay for insulin was established. Serum-insulin levels were log-normally distributed. Lean healthy subjects showed a ten fold range of insulin values. "Insulin resistance" was identified in consistently high insulin secretors, who showed correspondingly higher blood-sugar values. Normoglycaemic and borderline diabetic patients with vascular disease or obesity had elevated serum-insulin levels, but more severe diabetes was associated with a delayed and inadequate response to oral glucose. The one hour sample after 50 g. glucose identified most clearly the "insulin resistance" of normal subjects as well as the impaired insulin response of diabetics.

In population studies, the independent relationships of various factors with the "one hour" serum-insulin levels were delineated. Blood-sugar levels showed the strongest association with serum-insulin. The latter rose steeply with blood-sugar levels up to 180 mg/100 ml., but beyond this a progressive decline of insulin values occurred. Other major factors associated positively and independently with elevated serum-insulin levels were age, obesity, hyperuricaemia, and hypokalaemia. The insulin response to the standard glucose load varied inversely with height. Females had consistently higher levels than males.

Fasting serum-insulin levels were closely correlated with serum triglycerides both in normal subjects and those with coronary heart disease. Presumably insulin may have a physiological role in

promoting hepatic triglyceride synthesis.

Measurement of serum-insulin provided useful insight into possible mechanisms of disease; but was of limited use in discriminating diseased individuals. In the stages of diabetes mellitus, borderline hyperglycaemia was related to "insulin resistance", which may, in part, reflect varying vascular permeability. More severe hyperglycaemia was associated with a progressive impairment of insulin response, of undetermined cause, although hyperosmolality may contribute to this. Hyperinsulinism is possibly a "pre-diabetic" phenomenon.

In subjects with vascular disease in the population, the association with elevated serum-insulin levels was confirmed, particularly in the younger subjects with coronary heart disease and in hypertensives generally. Serum-insulin may usefully characterize the metabolic status of population samples, for healthy and active subjects showed low levels that did not rise until late age: whereas obese, hyperglycaemic, or vascular disease subjects had prematurely elevated levels, and an accelerated rise with age. The findings are compatible with the hypothesis that insulin has an aetiological role in the early genesis of atherosclerosis.

INDEX

CONTENTS

	<u>Page</u>
ABSTRACT 	1
INDEX 	3
PREFACE 	5
<u>CHAPTER 1</u> HISTORICAL REVIEW: circulating insulin in health and disease. The aims of the study	7
<u>CHAPTER 2</u> THE DOUBLE ANTIBODY IMMUNOASSAY OF INSULIN: assessment and modification of a method	48
<u>CHAPTER 3</u> THE NORMAL INSULIN RESPONSE TO GLUCOSE	80
<u>CHAPTER 4</u> SERUM-INSULIN IN PATIENTS WITH VASCULAR DISEASE	113
<u>CHAPTER 5</u> SERUM-INSULIN IN OBESE PATIENTS WITH AND WITHOUT DIABETES, AND THE EFFECT OF STARVATION	140
<u>CHAPTER 6</u> SERUM-INSULIN "ONE HOUR" AFTER GLUCOSE IN A TOTAL NATURAL COMMUNITY:	
Introduction	168
The "one hour" serum-insulin and its relationship to physiological variables	190
The "one hour" serum-insulin in health and disease	238

	<u>Page</u>
<u>CHAPTER 7</u> FASTING SERUM-INSULIN LEVELS IN SURVEY SUBJECTS	279
<u>CHAPTER 8</u> GENERAL SUMMARY AND CONCLUSIONS	302
<u>APPENDIX I</u> MULTIPLE REGRESSION ANALYSIS	312
<u>APPENDIX II</u> RELATIONSHIP BETWEEN SERUM-INSULIN AND BLOOD-SUGAR LEVELS IN HYPERGLYCAEMIC SUBJECTS	315
REFERENCES 	318

PREFACE

The clinical studies described in this thesis were conducted during a research assistantship to Professor Russell Fraser at the Royal Postgraduate Medical School in London (1964-1966); and the epidemiological studies during a research fellowship (1966-1969) at the University of Western Australia. All insulin assays reported were performed in my laboratory; about half were completed with technical assistance under my supervision.

I particularly wish to thank Professor Russell Fraser for his most helpful supervision and encouragement; and also Professors E. G. Saint and R. A. Joske for providing facilities and support in Western Australia. Assistance in the laboratories and wards of Hammersmith Hospital from Doctors Stefka Cevreska, Arthur Rubenstein, Iris Trayner, and David Wright is gratefully acknowledged. The population studies were conducted in collaboration with Dr Kevin Cullen (Georgiana Molloy Centre, Busselton), Professor David Curnow (Department of Biochemistry, Royal Perth Hospital), and Dr Michael McCall (Department of Medicine, Royal Perth Hospital), to each of whom I am greatly indebted. Dr Neil Cumpston coded the electrocardiograms; and Miss Margaret Summers and Dr David Jenkins undertook the blood lipid measurements. Technical assistance with the insulin assays by Mrs Penelope Power and Mrs Yukie Mak-Lee is acknowledged with gratitude. The excellent computing abilities of Mrs Clare Johnstone were a most vital contribution to the studies. For her energies and skills I am particularly grateful.

The assistance given by other colleagues is acknowledged in the appropriate parts of the text. The study of hypertensive patients on long-term diuretic therapy (Chapter 4) was specifically a collaborative project, planned jointly with

Drs Colin Dollery and Alasdair Breckenridge (see publications, below). Mr Norman Stenhouse, Director of the Raine Foundation Medical Statistics Unit (University of Western Australia) has given much helpful statistical advice with various aspects of the work. His assistance in the interpretation of multiple regression analysis, and his collaboration in devising the fitting of standard curves by the computer (Chapter 6) is recorded with gratitude.

The work was performed during tenure of a Thomas Saw Medical Research Fellowship (1964-1965); a British Medical Research Council Fellowship (1965-1969); and a Raine Research Fellowship in Arteriosclerosis (1966-1969).

Some of the work described in this thesis has been published in part, as follows:-

Welborn, T. A. and Fraser, R., (1966); the double antibody immunoassay of insulin, *Diabetologia*, 1, 211.

Welborn, T. A., Rubenstein, A. H., Haslam, R. and Fraser, R., (1966); the normal insulin response to glucose, *Lancet*, 1, 211.

Welborn, T. A., Breckenridge, A., Rubenstein, A. H. et al., (1966); serum-insulin in essential hypertension and peripheral vascular disease, *Lancet*, 1, 1336.

Breckenridge, A., Welborn, T. A., Dollery, C. T. et al., (1967); glucose tolerance in hypertensive patients on long-term diuretic therapy, *Lancet*, 1, 61.

Welborn, T. A., Johnstone, C. T. and Stenhouse, N., (1969); factors determining the serum-insulin response in a population sample, *Diabetologia*, 5, 263.

Timothy A. Welborn
Department of Medicine
University of Western
Australia

CHAPTER 1

HISTORICAL REVIEW

Insulin shares with its fellow hormones the ability "to arouse to activity" and this as a common pattern emerges in the history of its discovery. Endocrinology as a science began with observations on the effects of removal of ductless glands, and on the restoration of normal function by grafting or by the administration of extracts. Thus was insulin identified, and no account of insulin should fail to acknowledge however briefly the pioneers who contributed to this goal. Von Mering and Minkowski first achieved total pancreatectomy (1889) and thereby unmasked the anatomical origin of the diabetic state. Paul Langerhans had earlier identified the ductless islands within the exocrine pancreas (1869) and de Meyer named, by inference, their product "l'insuline" (1909). The triumphant Banting and Best captured the elusive chemical and demonstrated its remarkable ability to alleviate the previously fatal syndrome of diabetes mellitus (1922). The property of crystallization (Abel 1927) and the protein nature of insulin (Jensen 1932) were demonstrated, and subsequently the significance of zinc in the crystallization process (Scott 1934). The primary structure of the insulin molecule was ultimately elucidated by Sanger and his group (1955), the molecular weight of the monomer defined, and species differences in the amino acid sequence of the "A" and "B" chains determined.

"IN VIVO" BIOLOGICAL ASSAYS.

There was an immediate need to measure insulin when it became available for widespread therapeutic use. The potency of insulin preparations, initially determined by the hypoglycaemic response in diabetic dogs as in Best's laboratory, was assayed by measuring the reduction of blood-sugar of normal rabbits (Banting et alii 1922) and this procedure was subsequently standardized (League of Nations Health Organisation Report 1926). Four International Insulin Standards have now been prepared, and there is evidence to suggest that the International Unit has remained unchanged in value since 1935, with very little variation in potency since the First International Standard was produced in 1925 (Lacey 1967). The unit of insulin is currently defined as "the activity contained in 0.04167 mg. of the Fourth International Standard preparation" (World Health Organisation 1959) and is the amount that lowers the blood sugar levels of normal twenty-four hour fasted rabbits weighing 2 kilogrammes from 120 mg. to 45 mg. per 100 ml. within 5 hours, as assayed by a carefully controlled cross-over procedure.

The production of convulsions in fasting mice became an established alternative to the rabbit method for standardizing therapeutic insulin particularly in smaller laboratories, where the expenses of up to 198 rabbits per assay were difficult to meet. The dose of insulin was related to the proportion of mice observed to fall from a sloping screen within a standard time. The need to increase the sensitivity of assay procedures so that the smaller concentrations of insulin in tissues and body fluids might be measured led to the preparation of hypophysectomized-adreno-demedullated rats (Gellhorn, Feldman, and Allen 1941) in whom injection of 0.0001 units of insulin per 100 gm. body weight resulted in convulsions and coma. Using such an assay, the insulin content of normal human blood was reported to be 0.0002 units per ml., a

figure surprisingly close to present day estimates, although its validity was somewhat diminished by finding the same order of insulin-like-activity in extracts of potatoes and yams. Their demonstration that the plasma of fed dogs had approximately double the apparent insulin content of the plasma of fasted dogs suggested, nevertheless, that insulin was in fact being assayed. The specificity of the mouse-convulsion assay was further improved by the use of rotating screens that facilitated the fall of hypoglycaemic animals (Young and Lewis 1947). Thus the insulin content of extracts of human urine was determined (Mirsky et alii 1948): normal subjects excreted 0.16 ± 0.04 units per day, and diabetic subjects 0.07 ± 0.03 units per day - a minute clearance in view of the usual "replacement" dosage of insulin required of at least 20 to 40 units daily. Further modifications, including the induction of alloxan diabetes, to produce "ADH" rats (adrenomedullated, diabetic, hypophysectomized) with no possible interference from their endogenous insulin, and estimating the percentage fall in blood glucose, lowered the limits of detection to 0.125 milliunits (Anderson, Lindner, and Sutton 1947). Bornstein in 1948 reported the use of ADH rats to measure insulin in human plasma, and the sensitive range of his assay was from 0.05 to 0.5 milliunits (Bornstein 1948). In plasma, two hours after glucose, 14 normal persons had a mean insulin activity of 0.34 milliunits per ml. (range 0.24 to 0.40 milliunits per ml.) compared with a mean level of 0.23 milliunits per ml. (range 0.10 to 0.32 milliunits per ml.) in 13 diabetics who as a group were characterized by obesity and lack of ketosis. Another 15 diabetics had no detectable insulin activity in their plasma - this group showed weight loss and ketosis. Thus the clinical syndrome of diabetes was differentiated into two types on the basis of presence, or absence, of insulin action as measured by this method (Bornstein and Lawrence 1951, Bornstein 1953).

"IN VITRO" BIOLOGICAL ASSAYS.

The subsequent exploration of techniques for measuring the concentrations of circulating insulin in man involved the pursuit of sensitivity sufficient to detect the very low levels circulating in normal and diabetic subjects, specificity for the hormone derived uniquely from the beta cells of the pancreas, and convenience such that suitable numbers of samples could be processed. Biological assays that used the measurable effects of insulin on isolated muscle or adipose tissues were developed, and the immunological properties of insulin were exploited both in conjunction with the bioassays, and also later in the separate evolution of the radio-immunoassay. Since the results from each of these methods led to diverging concepts of the nature of insulin in blood, and of the aetiology of diabetes, their individual development will be reviewed.

RAT HEMIDIAPHRAGM ASSAY.

Incubation of rat diaphragms in physiological media results in glucose depletion of the media proportional to its insulin concentration. This system had been used for some time to investigate the effects of crystalline insulin solutions, but Groen et alii (1952) demonstrated its ability to detect insulin activity in normal human sera, at a concentration estimated to be in the range 62.5 to 625 micro-units per ml. The sera of insulin-treated diabetics, and of 2 subjects with "functional" hypoglycaemia contained insulin activity not appreciably different from that of normal subjects, whereas serum of a subject with an islet cell adenoma had five times the normal level. In contrast, ketotic diabetics' sera had no insulin activity. These authors attempted to exclude the possibility of insulin antagonists by obtaining appropriate "recovery" of the effect of crystalline insulin added to such sera. The specificity of the assay was supported by the evidence for cysteine inactivation of serum-insulin-activity; and by the demonstration of absent insulin activity in the sera of dogs

rendered diabetic and ketotic by total pancreatectomy. However, in the same year, an anti-insulin effect of normal human sera on the metabolic activity of rat diaphragm was reported (Marsh and Haugaard, 1952). Adopting more rigorous control of experimental conditions, Vallance-Owen and Hurlock (1954) assayed the plasma of fasting normal subjects, finding a range of insulin activity of 40 to 80 micro-units per ml. and a remarkably close correlation with the fasting glucose concentration. One hour after oral glucose loading, an increase of up to eight-fold of the fasting levels was observed (100 to 625 micro-units per ml.). In the whole plasma of mild obese diabetics, similar levels to the normal subjects were found. The activity of crystalline insulin added to the whole plasma of normal subjects and mild obese diabetics were quantitatively recovered.

Randle, using a similar assay found the mean insulin activity of normal human plasma, two and a half hours after a glucose load, to be 13.5 milli-units per ml. (range 9-22 milli-units per ml.) The plasma of acromegalics contained increased activity (40 - 284 milli-units per ml.) and that of hypopituitary subjects very low activity ;(0 - 3 milli-units per ml). He was careful to stress that "insulin activity in biological assays depended on the balance between substances having insulin-like action, (possibly growth hormone) and insulin-inhibitory actions (unidentified)". Variable sensitivity of the diaphragm assay was described within various laboratories, but of greater concern was the widely discrepant results for normal sera obtained between laboratories, for which no good explanation was found. When later positive evidence accumulated for the presence of a true insulin antagonist affecting the assay, the application of the technique to the measurement of levels in man generally ceased, although its use for 'in vitro' studies of the mode of action of insulin continued. One further useful finding however was that insulin activity increased several-fold in the plasma of

normal subjects and mild diabetics following tolbutamide administration, although tolbutamide had no effect alone on 'in vitro' muscle uptake, thus confirming in man the conclusions from animal experiments that this drug acts primarily by promoting the liberation of insulin (Vallance-Owen et alii 1959).

Plasma insulin activities in normal and diabetic subjects, using undiluted serum with the isolated rat diaphragm were determined by Seltzer and Smith (1959) both basally and 1 hour after a 100g. of oral glucose. The fasting levels of PIA in normal subjects and maturity onset diabetics were similar, but the post-glucose levels of PIA were such that the mild adult diabetics (tolbutamide-responders) showed a slightly diminished PIA whereas the more severe adult diabetics had a substantially impaired PIA found to be not significantly different from their fasting levels. Juvenile diabetics showed no PIA, neither fasting or after glucose. A delay in pancreatic secretory response, proportionate to the severity of the diabetes, was inferred and remains/^atenable thesis. In a further study, also using the rat diaphragm technique (Seltzer and Harris, 1964) the PIAs of various groups were studied during 5 to 7 days continuous glucose infusion, amounting to 700 g. daily. Surprisingly, normal subjects maintained normoglycaemia throughout (mean blood-sugar 91 mg. per 100 ml.) but this occurred by virtue of a five-fold increase in PIA., rising to ten-fold on the seventh day. Tolbutamide responding diabetics had levels of PIA, day for day throughout the test, that were closely similar to the normal subjects; but showed moderately severe hyperglycaemia (288 mg. per 100 ml.). The tolbutamide non-responding diabetics developed a progressive and more profound hyperglycaemia (530 mg. per 100 ml.); their PIA rose to a peak on the second day and thereafter declined to almost undetectable levels on the sixth and seventh days of the test. The study was designed "to telescope into a week, years of unremitting hyperglycemic stress" and did indeed demonstrate

in a dramatic manner the "exhaustion" of insulin-secretory reserve in moderately severe adult diabetics. The results were compatible with both the impaired responsiveness of peripheral tissues and a pancreatic beta cell defect, or possibly a combination of these factors.

THE SYNALBUMIN INSULIN ANTAGONIST.

Vallance-Owen, Hurlock and Please (1955) provided the first substantial evidence for the presence of an unidentified insulin antagonist in human plasma. Ketosis-prone, uncontrolled diabetics, with raised blood sugar levels, had plasma that failed to promote glucose uptake by the rat diaphragm, and the addition 'in vitro' to these plasma of large quantities of insulin (4000 micro-units per ml.) yielded no evidence of insulin activity. Plasma obtained from apparently similar insulin-requiring diabetics but controlled (with insulin) so that their blood sugar levels were in the physiological range showed definite insulin activity with values comparable to normal subjects. It was inferred that the circulating antagonist might be overcome by administration of sufficient insulin. The subsequent finding that insulin antagonism resided in the albumin fraction of the plasma proteins (Vallance-Owen, Denness, and Campbell, 1958) excluded insulin antibodies as being the cause of the inhibition. Employing the rat diaphragm technique as a method to detect antagonism of 1000 micro-units per ml. of insulin added 'in vitro' to the media, the albumin fractions (in contrast to whole plasma) of even normal subjects showed positive antagonism when present at the physiological concentration of albumin (3.5 to 5.5 g. per 100 ml.). Using albumin extracted from plasma at a standardized concentration of 1.25% the insulin antagonist was positive in groups of insulin-requiring diabetics, mild obese diabetics and subjects whose previous diabetic condition (associated with pregnancy, infection, or cortico-steroids) was latent; but

negative in selected normal subjects. The findings in diabetics and normal subjects were broadly confirmed and the lack of insulin antagonism on rat adipose tissue demonstrated (Lowy et alii, 1961; Alp and Recant, 1963). Subsequently, 19 out of the 28 non-diabetic subjects with cardiac infarction were shown to have positive (that is excessive) insulin antagonism in their plasma, compared with 6 out of 28 age and sex matched controls (Vallance-Owen and Ashton, 1963). The mode of inheritance of the factor (i.e. extracted albumin at 1.25% concentration inhibiting the activity of 1000 micro-units per ml. of insulin on rat diaphragm) in several diabetes-prone kindreds was shown to be by direct Mendelian dominance (Vallance-Owen, 1966). The distribution of the observed glucose uptakes by rat diaphragms, for the albumin samples obtained, shows almost complete separation into two groups - antagonistic and non-antagonistic. Such discrimination is rare in disease syndromes, excepting those due to hereditary enzyme deficiency(!).

The nature of the antagonistic factor is obscure and it has not been isolated. It can be separated from albumin for which it has affinity, is of low molecular weight (4000 or less), and it is heat-labile thus having similarities to the "B" chain of insulin. (Vallance-Owen 1965). Its presence in man may depend on pituitary and adreno-cortical function (Vallance-Owen and Lilley 1961), but this was not confirmed by Lowy et alii (1961). Using an entirely separate experimental approach, Young (1966) while investigating the seasonal variation in lipogenesis of mammals, demonstrated that human and other species' sera inhibited the intraperitoneal activity on the diaphragm muscle of insulin-injected rats 'in vivo' whereas the insulin effect on adipose tissue was not impaired.

The latter antagonist has well defined endocrine dependence in rats and appears to originate in the digestive

tract (Young and Benson 1966) but its identity with the synalbumin antagonist has not been established.

Clearly, a factor can be isolated from human plasma in association with albumin that inhibits insulin activity on rat muscle but not rat adipose tissue, 'in vitro' or 'in vivo'. This factor appears to be a "marker" for idiopathic diabetes, and possibly generalized vascular disease. But there is, as yet, no direct evidence to substantiate its physiological role in man, and thus its relevance to the action of circulating insulin must await its chemical identification and more precise measurement.

RAT ADIPOSE TISSUE ASSAY.

An assay for insulin using adipose tissue 'in vitro' was described by Martin, Renold, and Dagenais in 1958, utilizing the carbon dioxide or lipid production from glucose-1-¹⁴C by the isolated epididymal fat pads of albino Wistar rats. The method was extremely sensitive, the lower limit of detection being 10 micro-units of insulin per ml., and it was comparatively convenient since two standards and four unknown samples could be assayed simultaneously over one and a half days. At the outset it was acknowledged that the technique measured "insulin-like activity" (ILA) rather than true insulin in biological fluids, for it was already known that other hormones could mimic insulin's effects on rat adipose tissue, including oxytocin, prolactin, and glucagon (although only in considerably greater concentration, for a given effect, than insulin). Some reports of the total ILA in human serum appeared. Lyngsoe (1961) gave the mean fasting levels ($\pm$ one standard deviation) of ILA for normal subjects and for non-obese adult diabetics as 62 ± 38 , and 77 ± 73 micro-units per ml; compared with 168 ± 95 micro-units per ml. for obese adult diabetics, and 55 ± 47 micro-units per ml. for 16 juvenile

diabetics. The levels 90 minutes after glucose were approximately double the fasting values for ILA in each group. In the 75 normal subjects, no significant trends with age, sex or obesity were found. One disconcerting finding was that dilution of the serum samples generally led to considerable augmentation of the observed ILA values. Steinke et alii (1963) completed a large series of fasting ILA measurements in 56 controls, 42 prediabetics (children of two diabetic parents), 23 young and 10 middle-aged untreated diabetics. The mean fasting ILA levels ($\pm$ standard error) of serum diluted 1 in 4 were 91 ± 7 , 172 ± 10 , 165 ± 17 , 196 ± 27 micro-units per ml. respectively. Both these studies indicate the very wide range of results and the considerable overlap of values in health and disease states that are common to data obtained from biological assays. Nevertheless, the finding that juvenile diabetics may have at least normal, and possibly elevated, levels of ILA conflicted with the generally accepted belief that this condition was characterized by an absolute insulin deficiency, and led to speculation that a phase of hyperinsulinism may precede eventual failure of insulin secretion in the early stage of the disease. A smaller number of subjects in the report of Steinke et alii were studied during intravenous glucose tolerance tests; seven of the genetically "pre-diabetic" subjects showed a very gradual rise of circulating ILA to 180% of the fasting level by 1 hour; in contrast to normal subjects had a five-fold rise of ILA by 20 minutes. Thus a 'deficiency' of insulin may not become manifest unless provocative test is used that stimulates secretion.

Although serum ILA mimicked all of the effects of crystalline insulin on rat adipose tissue 'in vitro', reports that total pancreatectomy did not abolish its presence (Leonards, 1959; Goldberg and Egdahl 1961) and the failure of anti-insulin serum to inhibit its action (Leonards, 1959; Renold et alii 1960) led to a more detailed examination of its nature.

"NON-SUPPRESSIBLE" INSULIN-LIKE ACTIVITY (NSILA).

Froesch in 1961 initiated the term "non-suppressible ILA" to describe that fraction of serum retaining insulin-like action on the rat fat pad in the presence of excess anti-insulin serum - the synonym "atypical ILA" is also used (Samaan et alii 1962). The ILA from serum of dogs after pancreatectomy was entirely "non-suppressible" and its persistence during subsequent fatal keto-acidosis (Steinke et alii, 1962) provided strong evidence that it had an origin and biological activity other than pancreatic insulin. Considerable progress in elucidating the nature of NSILA has been made by Froesch and his colleagues (1967). A close similarity between the action of NSILA and crystalline insulin has been established, both on fat and muscle tissue 'in vitro'. However, in its native state, the molecular weight is in the range 70,000 to 150,000, and there is evidence in man that NSILA is restricted to the vascular compartment (Rasio et alii 1967). The origin, and biological significance, of NSILA is at present undetermined. Low levels in juvenile or lean adult diabetics have been reported (Samaan and Fraser 1963) but not confirmed (Froesch et alii 1963) and there seems to be general agreement that serum NSILA does not vary during the course of a glucose tolerance test. The only change in NSILA consistently obtained in man has been rising values during starvation (Samaan et alii 1965 Bolinger et alii 1965, Froesch et alii 1967).

"SUPPRESSIBLE" INSULIN-LIKE ACTIVITY.

"Suppressible" insulin like activity in serum is that part of total insulin-like activity, on the rat adipose tissue, that is inhibited by anti-insulin serum. Not unexpectedly, significant correlations between "suppressible" ILA and insulin as measured by immunoassay, have been reported (Sonksen et alii, 1965, Trayner et alii 1967). Serum "suppressible" ILA

risers promptly after a glucose load in normal subjects (Samaan and Fraser 1963) and there is a good correlation with blood glucose (Froesch et alii 1967). During oral glucose tolerance tests, diabetics, both juvenile-type and adult-onset, have normal or low fasting levels with a very sluggish or an absent rise after glucose. After pancreatectomy in dogs, the "suppressible" ILA disappears and diabetes ensues (Samaan et alii 1963; Rasio et alii, 1967). It seems very likely that "suppressible" ILA gives a measure of the true insulin content of serum, but the technique is tedious and the results obtained, in common with other biological assays, show variability and a lack of precision. Also the method is subject to artefact unless conditions are strictly controlled, for dilution of sera can result in considerable augmentation of the apparent insulin-like activity (Lyngsoe 1962; Power et alii 1965) as can increased osmolarity of the medium incubating the fat pad (Kuzuya et alii 1965).

"BOUND" AND "FREE" INSULIN

A separate approach by Antoniadou to the investigation of the nature of circulating ILA in man has led to the concept of "bound insulin" and "free insulin". The passage of serum through cationic exchange resins resulted in a loss of that part of ILA on adipose tissue that was associated with a basic protein, and thus appeared to be in a "bound" form. The remaining ILA, like crystalline insulin, was not retained by the resin and was called "free insulin" (Antoniades 1961). Both forms of "insulin" had ILA on rat adipose tissue whereas "bound insulin" was inactive on rat diaphragm unless previously incubated with adipose tissue extracts. It was presumed that the latter liberated "free" from "bound insulin". Serum samples were therefore assayed by prior incubation with adipose tissue extracts, and then separately eluted from

cationic exchange resins. After pH adjustment, dialysis, and lyophilization, the eluates were incubated with rat diaphragms 'in vitro'. In fasting non-diabetic subjects, "bound insulin" was the predominant form in serum, but after intravenous glucose there was a rapid appearance of "free insulin" and a sharp decline of "bound insulin". In contrast, new diabetics had substantial ILA that remained largely in the "bound" form throughout the glucose tolerance tests (Antoniades et alii, 1962). However it can be derived from these workers data that total ILA ("bound" plus "free") shows a progressive and marked reduction from 10 to 60 minutes after intravenous glucose in non-diabetic and diabetic subjects (Antoniades et alii, 1961) a finding that conflicts with most studies. The substantial manipulation of serum samples prior to assay must raise doubts as to the validity of the final estimate of ILA, particularly since the rat diaphragm assay is known to lack sensitivity and specificity. The presence of an antagonist to insulin's action on muscle may partly account for the results (Ensinck et alii 1965). However, attempts to replicate Antoniades' techniques have failed to show conversion of "bound" to "free insulin" (Gener-Segui and Wolfe, 1966; Mead et alii 1968) and the production of immunologically identified insulin has not been demonstrated in plasma incubated with adipose tissue extract (Berson and Yalow 1964). As with the other insulin-like and insulin-antagonistic substances detected by biological assays, reservations must be expressed about the significance of "bound" insulin until evidence for its physiological importance is obtained.

SOME CONCLUSIONS FROM BIOLOGICAL ASSAYS.

Despite the poor specificity of bioassays, and the occurrence of a number of factors of obscure origin found to be synergistic or antagonistic to insulin's action, some consistent features of various studies enabled general conclusions to be derived. Juvenile-type or lean diabetics showed

a marked deficiency of circulating "insulin" whereas maturity-onset or obese diabetics had at least normal and often elevated levels of insulin-like activity, confirming longstanding clinical and pathological observations. When provoked by glucose loading, the insulin secretory response of diabetics or prediabetics appeared to be impaired, but tolbutamide was shown to augment the levels of insulin-like activity in some cases of diabetes. Further useful information from bioassays pertaining to the possible binding of insulin by protein fractions is reviewed in a subsequent section.

DEVELOPMENT OF RADIO-IMMUNOASSAY TECHNIQUES.

The ultimate development of radio-immunoassays for insulin heralded a period of vast progress in research in this field, for these techniques possessed exquisite sensitivity and apparent specificity, and were rapidly applied to clinical problems. The major advances leading to this achievement were recognition of the antigenic properties of crystalline insulin and development of methods for labelling insulin with radio-active iodine.

The antigenicity of commercial insulin, of beef or pork origin, was long suspected from observations of anaphylaxis in insulin-treated guinea-pigs (Banal and Roux 1931), and from the development of insulin resistance in patients receiving repeated insulin "shock" therapy (Banting et alii 1938). Antibodies to insulin in rabbits were demonstrated by complement fixation (Wasserman et alii 1940), and evidence for species specificity of insulin antibodies was provided by Moloney and Covall (1955). The specific identification of antisera to beef insulin was achieved by use of insulin-conjugated sheeps red cells, with haemolysis or haemagglutination as the end point of antigen-antibody combination; and the cross-reaction of these anti-beef insulin antibodies with human insulin suggested an application in the assay of serum insulin (Arquilla and

Stavitski 1956). The use of haemagglutination or haemolysis subsequently fell into disrepute for immunological assays because of the phenomenon of non-specific inhibitors, and the lack of reproducibility between laboratories (Fraser and Hartog 1962).

Labelling of insulin with ^{131}I Iodine (Ferrebee et alii 1951) did not impair biological activity and the techniques were further developed (McFarlane 1958, Greenwood et alii 1963) to enable the preparation of labelled protein hormones of suitably high specific activity (up to 300 μC per $\mu\text{g.}$). It has been shown that Insulin- ^{131}I retains full biological activity 'in vivo' or 'in vitro' unless the average incorporation of total iodine exceeds one atom per molecule of insulin, whereas the ability to bind with specific insulin antibodies is fully retained with an average incorporation of total iodine of up to 6 atoms per molecule of insulin (Izzo et alii, 1964).

Using Insulin- ^{131}I administered intravenously to human subjects the labelled insulin in normal plasma, when subjected to paper electrophoresis, remained at the site of application; whereas labelled insulin in the plasma of subjects who had received insulin injections for several months migrated with the plasma globulins (Berson et alii, 1956). Thus chromatoelectrophoresis for separation of antibody-bound from free Insulin- ^{131}I originated. In a similar study of induced insulin antibodies in diabetic subjects, a rabbit antibody to human gamma globulin was used to precipitate the insulin-antibody complexes, foreshadowing the "double antibody" techniques used for immunoassay (Skom and Talmage 1958).

The use of an antibody as the "detector" of insulin in biological fluids was attractive because of the well known chemical specificity of antibodies. This however created problems, for the readily available human antibodies to bovine/porcine insulin reacted weakly with human insulin (Berson and Yalow 1959). It was eventually found that batches of guinea pigs

immunized with beef or pig insulin would yield sera from one or two animals that were strong binders of labelled bovine insulin but with which human insulin competed sufficiently to provide suitable sensitivity for measurement of levels in man.

PRINCIPLES OF RADIO-IMMUNOASSAY.

Radio-immunoassay is a derivative assay based on the principles of isotope dilution. Thus:

- (1) When a fixed concentration of Insulin- I^{131} is reacted with a fixed concentration of specific insulin antibody, the antibody will bind a proportion of the Insulin- I^{131} according to the **Law** of Mass Action (Berson and Yalow 1962),
- (2) If the above system contains a further variable concentration of unlabelled insulin, the antibody will bind Insulin- I^{131} and unlabelled insulin in the same proportion as their initial concentrations prior to binding.
- (3) Increasing the concentration of unlabelled insulin will result in a decrease in the proportion of Insulin- I^{131} antibody-bound.
- (4) The proportion of Insulin- I^{131} antibody-bound at various known concentrations of unlabelled insulin provides a standard curve from which the concentrations of unlabelled insulin in unknown samples can be derived.

The assumption is made that Insulin- I^{131} reacts with antibody in an identical fashion to the homologous unlabelled insulin, and this is borne out by experimental evidence (Berson et alii, 1964; Izzo et alii, 1964) although challenged by those using haemagglutination techniques to determine integrity of antigenic insulin (Arquilla et alii, 1968). Furthermore, it is desirable for the valid comparison of "unknown" with "known" insulin concentrations that both are the same species insulin, for although human insulin will cross react with suitable antisera to beef or pig insulin, the standard curves for given

concentrations of each insulin may be dissimilar (Berson and Yalow 1960). Finally, when using the various techniques of radio-immunoassay for estimation of insulin content of serum (or plasma) it must be established that the protein or other constituents of the unknown samples do not interfere with the immuno-chemical reactions of the assay at any stage. The assay of serial dilutions of sera, and of sera with and without known amounts of standard insulin added, provide checks for such possible artefacts.

TECHNIQUES OF RADIO-IMMUNOASSAY

Many different procedures have been elaborated for the radio-immunoassay of insulin, all of which make use of the principles outlined above. The major difference in the methods described relate to the method of separation of the antibody-bound insulin from the free hormone. The first definitive report of an assay procedure capable of measuring endogenous human plasma insulin utilized paper chromato-electrophoresis for this separation (Yalow and Berson 1960). Antibody-bound insulin migrates with plasma proteins towards the anode whereas the unbound insulin absorbs to the paper at the site of application. The partition of Insulin-I¹³¹ can be quantitated by scanning the paper strips for radioactivity. A graph of two peaks is obtained and the amount of Insulin-I¹³¹ contributing to each peak is assessed by planimetry. This method has the considerable advantage of demonstrating clearly the separation of antibody-bound from free hormone for each sample assayed, so that artefactual measurements resulting from incomplete separation are virtually impossible. However, paper chromato-electrophoresis entails the use of Insulin-I¹³¹ of high specific activity (75 to 300 milliCuries per mg. insulin) to obtain suitable counting of radioactivity, and restricts the total volume of the assay mixture to 0.5 ml. Incubation damage of high specific activity Insulin-I¹³¹ occurs particularly in the presence of plasma

volumes exceeding 50 μ l, producing labelled components that bind to plasma proteins, and necessitating the inclusion of control tubes to evaluate and correct for the extent of this artefact. In an otherwise elegant and reliable technique this is a comparatively minor drawback; nevertheless, the performance of multiple electrophoreses, the scanning and counting of paper strips and their subsequent planimetry, dictate the provision of specialised equipment and a high level of manual competence, and automation of these various stages is difficult.

"Double antibody" techniques for radio-immunoassay are more convenient and more popular. Isolation of the antibody-bound insulin is achieved by precipitating the guinea pig anti-insulin serum with a second antibody, for example rabbit anti-guinea pig serum or rabbit anti-guinea pig gamma-globulin. The precipitate, containing the antibody-bound insulin, is separated from the free insulin in the supernatant by centrifuging and decanting (Morgan and Lazarow, 1963) or by microfiltration through millipore membranes (Hales and Randle, 1963). The major disadvantage of these methods is the lack of certainty that complete separation of antibody-bound hormone is achieved, particularly since human serum proteins may interfere with the precipitating reaction. The advantages are those of technical simplicity and the use of equipment (centrifuge, well-type crystal or liquid scintillation counter) usually available in a large hospital or laboratory complex.

Other modifications of the assay procedure include the measurement of insulin in acid-ethanol extracts of sera with sodium sulphite precipitation of the insulin-antibody (Grodsky and Forsham 1960); the use of Sephadex (Frohman and Lebovitz 1965, Banerjee 1962) or of dextran-coated charcoal to separate the small molecular weight "free" insulin from the antibody-bound insulin (Herbert et alii, 1965). Resin-binding of antibody-bound insulin (Meade and Klitgarde, 1962) has also been employed. Most of these share the disadvantage of the

double antibody techniques that the presence of serum proteins may interfere with the separation of antibody-bound hormone to a greater or lesser extent, rendering the comparison of results against buffer solutions of standard insulin invalid unless carefully standardized.

DOES RADIO-IMMUNOASSAY MEASURE 'ALL' INSULIN?: THE NATURE OF INSULIN IN BLOOD:

The question as to whether radio-immunoassay provides an "absolute" measurement of circulating insulin will remain unresolved until the latter substance is specifically characterized and the differences in structure (if any) from extracted pancreatic insulin directly identified. Two major considerations to be resolved are, first, whether all the insulin in blood is available for reaction with specific insulin antibody; and second, whether the assay is specific for insulin in the presence of the myriad proteins, hormones, and enzymes that also circulate in blood. The sensitivity of the assay is not disputed, for the earlier reports indicate that standard methods are capable of detecting 1 to 5 μ U per ml. of solutions of crystalline insulin and that this sensitivity can be improved ten-fold by suitable manipulation of the concentration of reactants (Berson and Yalow 1964).

Evidence for the nature of insulin in blood from electrophoretic studies is summarized in Table 1A and 1B. The localization of endogenous insulin-like-activity in both the albumin - α_1 and the β - γ globulin zones is an almost constant finding, but true insulin, as specified by suppression with anti-insulin serum or augmentation by glucose loading, appears to be associated only with the former serum proteins (Table 1A). The results of localizing Insulin- I^{131} added to serum (Table 1B) are more variable, but in general, radioactivity has been discerned in the

TABLE 1.

A. LOCALIZATION OF INSULIN-LIKE ACTIVITY BY ELECTROPHORESIS OF NORMAL HUMAN SERA

Investigators	Methods		ILA in Serum Protein fractions					
	Serum fractionation	Insulin assay	pre-albu min	albu-min	α_1	α_2	β	γ
Biegelman (1958)	Curtain electrophoresis on paper	Mouse hypoglycaemia	-	-	-	-	+	+
Bolinger et alii (1959)	Starch column electrophoresis	Rat diaphragm	-	-	-	-	+	$\pm$
Taylor and Randle (1959)	Zone electrophoresis on cellulose	Rat diaphragm	-	² +	² +	-	² +	² +
Ditscheneit et alii (1964)	Polyvinyl chloride powder electrophoresis	Rat adipose tissue	-	³ +	³ +	-	⁴ +	⁴ +
Lyngsø (1964)	Electrophoresis	Rat adipose tissue	-	^{1,3} +	^{1,3} +	-	^{2,4} +	^{2,4} +
Berson and Yalow (1966)	Starch gel electrophoresis	Radio-immuno-assay	¹ +	¹ +	-	-	-	-

ILA

* ¹ = wholly suppressible by anti-insulin serum.

* ³ = ILA rises after glucose loading.

² = incomplete or absent suppression of ILA by anti-insulin serum.

⁴ = no rise of ILA after glucose loading.

* (i.e. probably insulin)

(i.e. probably not insulin)

TABLE 1 (Contd)

B. LOCALIZATION OF INSULIN - I¹³¹I ADDED TO NORMAL HUMAN SERA OR PLASMA

Investigators	Method of Serum Fractionation	Serum Protein Fractions					
		Pre-albu-min	Al-bu-min	α_1	α_2	β	γ
Berson et alii (1956)	Insulin-saturated paper chromatography	-	+	+	+	-	-
Berson and Yalow (1956)	Starch block electrophoresis	-	+	+	-	-	-
Clausen (1963)	Immuno-electrophoresis on agar	-	-	-	+(M)	+(M)	-
Mitchell (1963)	Paper chromatography	-	-	+	+	+	-
Prout (1964)	Immuno-electrophoresis on agar	-	-	+	-	-	-

(M) = Macroglobulin

α_1 globulins or the α_2 globulins. However, ultracentrifugation (Berson and Yalow 1962, Chao et alii, 1965) and gel filtration techniques (Chao et alii 1965, Poffenbarger et alii 1968) of both endogenous and labelled crystalline insulin indicate that both forms of insulin exist in plasma as substances of molecular weight considerably less than albumin and probably in the range 6,000 to 18,000.

Reviewing all of the above evidence it would appear that insulin has an association with plasma proteins under certain experimental conditions. Such binding must be weak and reversible since endogenous serum insulin or Insulin-I¹³¹ added to serum is adsorbed with ease to glassware, paper, and cellulose (Yalow and Berson 1960) and acid-ethanol extraction of sera reveals no "hidden" immunochemical insulin previously inaccessible to antibody (Berson and Yalow 1966; Karam et alii, 1963; Dash and Lindsay 1967). Specific anti-insulin serum is able to bind with ease all the Insulin-I¹³¹ added to serum without competition (Prout 1964). Insulin anti-serum inhibits the action of crystalline insulin on isolated fat or muscle tissue 'in vitro'; and 'in vivo' in many species, the injection of anti-insulin serum produces immediate evidence of acute insulin deficiency (Moloney and Coval 1955; Wright 1961). Thus it seems that the antibody has access to all of the antigenic and all of the biologically active insulin in serum.

SPECIFICITY OF THE RADIO-IMMUNOASSAY.

The specificity of the radio-immunoassay for insulin is supported by many observations that insulin so measured exhibits identical behaviour to that of crystalline or of Insulin-I¹³¹ added to plasma in all physicochemical systems explored. Also in numerous physiological and clinical studies the rise and fall of levels of immuno-reactive insulin (IRI) in the plasma of man and animals corresponds to the expected behaviour of the pancreatic hormone. Much of this evidence has

been exhaustively reviewed (Yalow and Berson, 1960, 1964; Berson and Yalow, 1962) and is summarized as follows with additional references.

1. Similarity of physico-chemical properties of plasma IRI and crystalline or Insulin-I¹³¹:

- (a) Cysteine, which destroys crystalline insulin by disrupting disulphide bonds, when incubated with plasma causes IRI to disappear.
- (b) Plasma IRI, crystalline insulin, and Insulin-I¹³¹ show identical sedimentation rates on ultracentrifugation.
- (c) Plasma IRI and Insulin-I¹³¹ show very similar electrophoretic mobilities on starch block or starch gel media (small differences have been attributed to the iodine content of Insulin-I¹³¹).
- (d) Plasma IRI and crystalline insulin have corresponding elution volumes by gel filtration (Poffenbarger et alii, 1968).

(NB: Insulin does not exhibit many remarkable physico-chemical properties).

2. Expected behaviour of plasma IRI in physiological studies:

- (a) In non-diabetic subjects plasma IRI rises promptly from low fasting levels after oral or intravenous glucose administration or after intravenous tolbutamide (Yalow et alii, 1960, Samols 1964).
- (b) The "half lives" of plasma IRI, intravenously injected insulin, and Insulin-I¹³¹ show generally corresponding values.
- (c) IRI after an intravenous glucose stimulus equilibrates rapidly between plasma and lymph, showing a closely similar partition rate to inulin of molecular weight 5500 (Rasio et alii, 1967).

3. Expected behaviour of plasma IRI in pathological states:

- (a) Endogenous plasma IRI is present in very low concentrations in established juvenile diabetics, who have been shown to have very little extractable pancreatic insulin (Wrenshall et alii, 1952).
- (b) Endogenous plasma IRI is virtually absent after pancreatectomy in man (Egdahl and Goldberg 1962).
- (c) The majority of subjects with hypoglycaemia due to islet cell adenomas have elevated plasma IRI in at least one of several fasting samples (Samols and Marks, 1963).

4. Chemical specificity of anti-insulin sera used to measure IRI:

Provided that the assay method enables complete separation of antibody-bound from unbound insulin even in the presence of plasma proteins, it is generally agreed that radio-immunoassay is the most specific measurement for circulating insulin yet devised.

- (a) The degree of discrimination shown by guinea pig antisera for different species' insulin with only minor differences in amino acid sequence suggests very high immunochemical specificity.
- (b) True cross-reactions of insulin antisera with other hormones have not been reported. Glucagon is known to be a trace contaminant of insulin extracted from the pancreas, and there is a theoretical basis to suggest a similarity in tertiary structure of the two hormones (Schuster 1966). However the immunoassayable insulin of crystalline glucagon preparations is minute, amounting to less than 0.1 percent by weight (Yalow and Berson, 1961). Human growth hormone does not interfere with the insulin assay (Wright, et alii, 1967).

- (c) The only substances known to cross react substantially with insulin antisera are closely related to insulin. Thus enzymic derivatives, desoctapeptide and desalanine insulins, may interfere with the assay, but these are not known to occur physiologically. Native A chain of insulin can have 2 to 3 percent of the activity of whole insulin by immunoassay, a possibly significant cross-reaction in view of the now known concentration of circulating A chain (Meek et alii 1968). "Pro-insulin" and "abnormal insulins" are also possible substances with immunological similarities to insulin and are discussed below.
- (d) An insulin-like effect of pancreatic proteins on biological assays and immunoassays has been reported (Reiser and Reiser 1964; Pruitt et alii, 1966). In particular, chymotrypsin at a minimum concentration of 7×10^{-6} M, produces a significant effect on the immunoassay possibly by hydrolyzing labelled insulin; however this is more than ten times the physiological concentration of the enzyme in serum, and is thus unlikely to be a source of artefact.
- (e) The reasonably close correlation of insulin concentrations in serum measured by immunoassay compared with "suppressible" ILA measured on rat adipose tissue in vitro (Sonksen et alii 1965) provided the necessary reassurance that what is assayed as IRI has corresponding biological activity.

All available evidence thus suggested that the immunoassay for insulin had almost absolute specificity for the hormone as identified from purified pancreatic extracts. The possibility that precursors, or degradation products or chemically different forms of insulin register in this assay system as insulin, when in fact these substances have impaired biological activity, requires careful consideration.

ABNORMAL INSULINS.

An abnormal insulin in juvenile diabetes mellitus has been proposed (Elliott et alii, 1965). Insulin as isolated from serum by gel filtrations, and reversible complexing with guinea pig anti-insulin serum, was submitted to the action of crude rat muscle insulinase. Insulin from the sera of juvenile diabetics was very considerably more resistant to destruction than either insulin from normal persons or crystalline insulin preparations and hence, perhaps, was structurally different. The grounds for caution in accepting these investigators' hypothesis perhaps depend on the extensive physico-chemical manipulation of endogenous insulin reported, the dissimilar final concentrations of purified endogenous insulin in disease and control groups prior to assessing "destruction rate" by a standard concentration of insulinase, and the possibility of artefact in the measurement of insulin by double antibody immunoassay for which few details are given. Further collateral evidence for the existence of "abnormal" insulin in juvenile diabetes would be desirable to support this interesting hypothesis. A recent report of the amino acid constitution, and also the biological and immunological properties, of insulin crystallized from pancreatic extracts of diabetic and of non-diabetic patients at autopsy indicates the complete identity of the insulins so tested (Brunfeld et alii, 1969), and thus the primary structure of the ethanol-extracted hormone is likely to be the same.

PROINSULIN.

Proinsulin has been identified as the probable precursor of insulin in the beta cells of mammalian pancreas. Its existence has been confirmed by delineation of the amino acid sequence of bovine and porcine proinsulins and its structure found to be a single polypeptide chain, with molecular weight about 9000. This is cleaved intracellularly by a

proteolytic mechanism to yield insulin. Adult islet cell tissues contain a preponderance of insulin rather than proinsulin, thus the latter appears to be a true biosynthetic precursor rather than a storage form of the hormone (reviewed by Steiner et alii, 1968). This concept contrasts with earlier suggestions that separate synthesis of A and B chains occurred prior to their linkage (Humbel 1965). Proinsulin is stated to have little biological activity, exhibiting about 2 percent of the ILA of insulin in 'in vitro' assays. However human proinsulin can react strongly with insulin antisera, and a preliminary report of the occurrence of proinsulin in human serum and urine has been published (Rubenstein et alii 1968). Relatively high levels of proinsulin have been found in the serum of one subject with insulinoma and one with untreated juvenile diabetes, conditions that possibly have in common a near maximal secretion of available beta cells. Clearly, proinsulin secretion may be important in diabetes where "insulin" is identified immunologically but there is evident lack of biological activity. The small concentrations of proinsulin, compared with insulin, in pancreatic islet tissues, perhaps indicates that in health the circulating levels of this substance are similarly low. Nevertheless, firm statements about the specificity of immunoassay of insulin in body fluids must await the determination of the coexisting proinsulin concentrations in health and disease. Also, where possible, the extent of interference of proinsulin with individual immunoassay techniques should be quantitated.

"BIG" INSULIN.

Following the discovery of proinsulin, a further examination of the state of circulating insulin led to the identification of two forms of insulin in human plasma (Gorden and Roth, 1969). Plasma filtered on dextran gels showed distinct fractions of immunoreactive insulin, "big" insulin and "little" insulin. "Little" insulin had a similar elution volume to

crystalline pork insulin and also identical quantitative reaction on dilution with antiinsulin serum. "Big" insulin did not show completely identical cross-reactivity with anti-insulin serum. Both components are secreted by the pancreas, but have a different time relationship to glucose loading. Thus in normal subjects after a standard 100 gm. oral glucose load, "little" insulin shows a brisk increment in the first hour and a more gradual decline towards fasting levels, whereas "big" insulin, which accounts for an estimated 0 to 5% of total immuno-reactive insulin basally, shows a gradual rise over 1½ to 2 hours, reaching 5 to 28% of the total "insulin" at this time. The inference seems reasonable that "little" insulin represents the secretion of stored insulin, and "big" insulin is newly synthesized and immediately released material. Their preliminary reports from a few studies state that lean adult diabetics (with hyperinsulinism) show no distinct difference in the proportions of the two forms of insulin. Obese subjects, diabetic or otherwise, and starving lean normal subjects, have a disproportionate increase in "big" insulin. It seems possible, even probable, that proinsulin and "big" insulin are the same substance.

THE SECRETION AND FATE OF INSULIN.

The use of suitable preparations of labelled insulin, and the development of the immunoassay technique, gave great impetus to physiological studies of insulin production and disposal. Thus much information has been gained about the secretion of insulin. One major stimulus for insulin release has long been known to be glucose (Anderson and Long, 1947). The existence of a separate intestinal factor was postulated by several groups (McIntyre et alii 1964; Elrick et alii 1964; Perley and Kipnis 1967) after demonstrating that the oral or jejunal administration of glucose yielded much greater levels of plasma IRI than intravenous glucose although hyperglycaemia was of equal magnitude.

Subsequent studies in man using purified animal secretin, (Boyns et alii, 1967) pancreozymin and possibly gastrin (Dupré et alii 1969) indicated that each of these substances was capable of promoting insulin release and of enhancing the serum-insulin response to glucose or amino acids. "Gut-glucagon" a further substance of probable intestinal origin, has been shown to rise during glucose absorption, and resembles pancreatic glucagon immunologically (Samols et alii 1965; Lawrance 1966). Pancreatic glucagon itself has been found to be a major stimulus for insulin secretion (Crockford et alii 1965). While it appears that "gut-glucagon" following glucose absorption and pancreozymin following amino acid absorption may have specific hormonal roles in augmenting insulin secretion (Unger and Eisenstraut 1969) clarification of their physiological importance is required. No reports are available yet of their relevance to disease. Adrenaline and noradrenaline promptly inhibit insulin secretion by acting on α -adrenergic receptors (Porte 1967) but the significance of this in disease states (apart from the diabetes of pheochromocytoma and of extreme stress) is probably small. Physiological nutrients capable of evoking insulin secretion include all ingested carbohydrates that yield glucose or related hexoses after digestion and absorption (Grodsky et alii 1963) and amino acids ingested as such or following hydrolysis of protein (Floyd et alii 1964; Rabinowitz et alii 1966). There is conflicting evidence in man as to whether ketones or free fatty acids stimulate beta cells (Madison et alii, 1964; Ruderman et alii 1969). A further possibility with regard to a secretory stimulus is that the liver in handling portal vein glucose, or other nutrients, elaborates a metabolite that enhances insulin secretory response. Earlier suggestive evidence in this direction (Balsano et alii 1964; Goetz et alii 1964) has had no detailed support yet.

INSULIN AND THE LIVER.

Insulin, secreted into the portal vein, encounters the liver. Studies using Insulin-I¹³¹ suggest that 40 to 50 percent of portal vein insulin is sequestered by the isolated perfused rat's liver (Mortimore et alii 1949) and by livers of anaesthetized dogs (Madison et alii 1959). In fasting human subjects with portal systemic shunts or severe liver disease, the proportion of arterial immuno-reactive insulin removed by the liver was 20 to 50 percent with a mean of 40 percent (Samols and Ryder 1961). The latter study showed that the liver fixation of endogenous and exogenous insulin was closely similar, and remained a constant proportion of the arterial insulin over a wide range of concentration. Although glucose diffuses freely into liver cells, and does not require a specific transport system (Cahill et alii 1958), the liver cell is nevertheless exquisitely sensitive to insulin, and responded by inhibiting glucose production and also increasing actual uptake (Madison et alii 1959, deBodo et alii 1959). The liver contains a specific insulin degrading enzyme, glutathione insulin transhydrogenase (Tomizawa and Halsey 1949; Katzen et alii 1963) that cleaves the molecule into A and B chains. To what extent intracellular insulin in the liver is involved in metabolic activity or degradation is uncertain, but 'in vivo' studies have located labelled and immunoreactive insulin within the liver cell in mitochondrial and microsomal fractions although the insulin lacks biological activity (Beck et alii 1966).

INSULIN AND THE KIDNEYS.

The kidneys also play a significant role in the removal of insulin from blood. Retarded degradation of Insulin-I¹³¹ has been demonstrated in nephrectomized animals (Elgee and Williams 1954), and in human subjects with severe renal disease (O'Brien and Sharpe 1967). Further studies with labelled insulin suggested that the concentration of insulin in the renal vein was 10 to 45

percent lower than arterial insulin levels (Lee 1957 et alii 1966) although somewhat less than 3 percent of radioactive protein appeared in the urine (Elgee et alii 1954; Berson et alii 1956). These data accord well with immunoassay findings that the renal arteriovenous difference in insulin concentrations for normal subjects was 30 to 40 percent (Chamberlain and Stammers 1967; Rubenstein and Spitz 1968) and that insulin found in the urine did not exceed 1 to 5 percent of the filtered load in health, although with severe renal tubular disease, urinary insulin clearance approaches the glomerular filtration rate. It is probable that insulin undergoes enzymatic degradation intracellularly in a similar manner in both kidney and liver (Beck et alii 1966). The precise influence of insulin on renal glucose balance is not known.

INSULIN AND PERIPHERAL TISSUES.

Insulin clearance by limb tissues is generally taken to represent its utilization by muscle and adipose tissue, the major peripheral targets of insulin action. In human subjects (Samols and Ryder 1961) the femoral arteriovenous differences in insulin concentrations were variable, ranging from 5 to 28 percent, with a mean of 20 percent, of the basal arterial level, by immunoassay. Using the human forearm preparation, Butterfield and colleagues (1963) found a muscle fixation of 13 percent of Insulin- I^{131} infused intra-arterially in normal lean subjects. There appeared to be a strong quantitative relationship between the amount of insulin fixed and the 'estimated' plasma insulin concentration. In addition the insulin fixation of forearm muscle was strictly proportional to the observed glucose uptake. Although juvenile diabetic subjects, some of whom had not previously received insulin, showed a much lower insulin fixation of 1.3 to 3.8 percent, the glucose uptake plotted against insulin fixation followed a similar correlation to that of normal subjects.

Thus the insulin sensitivity of diabetic muscle was related entirely to lack of tissue binding of insulin. Similar considerations applied to the muscle insensitivity of obese subjects (Butterfield, 1965). It is calculated that a molecule of insulin, when fixed to muscle, promotes the uptake of 2×10^6 molecules of glucose in normal, or obese, or diabetic subjects. Adipose tissue in vivo is probably substantially less sensitive than muscle to circulating levels of insulin, since its blood flow is comparatively small and arteriovenous differences across superficial forearm fat have been observed to be less than across the muscle compartment (Rabinowitz and Zierler 1962). There seems general agreement, however, that for insulin action, "tissue fixation" of insulin must occur as first proposed by Stadie and Colleagues (1952) from experiments 'in vitro' using rat diaphragm, and confirmed by Ball and Jungas (1964) using 'in vitro' rat adipose tissue, and more recently demonstrated 'in vivo' by Rafaelson (1966).

A recent report of experiments using the human forearm evaluated muscle glucose uptake in normal lean subjects during sustained intravenous glucose infusion. A very close relationship existed between arterial plasma insulin concentrations, measured by immunoassay, and glucose uptake, over a wide range (Christensen and Ørskov 1968). This confirms earlier conclusions of Butterfield who used labelled insulin. The correlation between the serum-insulin level and glucose uptake was particularly close in any one individual, but between individuals the slopes of the lines were variable, suggesting inherent differences in insulin sensitivity or fixation, from subject to subject. One of the not unimportant findings emerging from the experimental data is that immaculate physiological evidence is provided for the validity of serum immuno-reactive insulin levels in man. It was also of interest that arterial serum insulin concentrations exceeding 200 μ U per ml. produced no further glucose uptake in a small number of subjects, implying possible saturability of the insulin-responsive transport system.

INSULIN SECRETION AND UTILIZATION RATES.

Insulin has a brief sojourn in the blood. The early reports of a half-life in the circulation of 20 to 50 minutes with a mean of 35 minutes, were based on studies using high specific activity Insulin-I¹³¹ (Berson et alii 1956) but recent studies of decline of endogenous insulin levels (Samols and Marks 1966) and of exogenous insulin (Orksov and Christensen 1966; Tomasi et alii 1967) give a half-life of well within 6 to 15 minutes. The studies of both Berson and of Tomasi indicate no significant difference between the disappearance rates of insulin in normal and in non-insulin treated diabetics. Thus, despite marked differences observed in muscle fixation of insulin between normal and diabetic subjects it would appear that overall insulin degradation at other sites proceeds at similar rates, and the levels of insulin measured in diabetics do not reflect differences in insulin removal rate.

Measurement of insulin secretion rate in man is likely to be very difficult because of insulin's release into the portal vein and the liver's capacity to remove a large proportion of the secreted load. The rate of insulin production into systemic circulation plasma has been estimated. Stern and his colleagues (1968) studied a suitably large group of normal and diabetic subjects under steady state conditions of fasting or continuous intravenous glucose infusion. The "irreversible loss rate" of tracer Insulin-I¹³¹ from the plasma compartment was measured in conjunction with plasma glucose and immunoreactive plasma insulin concentrations. A direct linear relationship was found between the plasma insulin concentration, (range 10 to 210 μ U/ml) and the insulin irreversible loss rate (range 1200 to 37,818 μ U/minute). Thus the insulin removal mechanism, in normal and diabetic subjects, does not become saturated over this range of insulin concentrations. Furthermore, assuming a true steady state, insulin production must have equalled insulin irreversible loss and the changes in

plasma insulin concentration therefore were linearly related to the rate of insulin secretion. This provides additional compelling support for the physiological meaningfulness of insulin levels measured by immunoassay, particularly since the subjects studied were selected to provide a wide range of blood-sugar levels and body weight. Diabetes and obesity did not appear to have influenced the close relationship obtaining between the venous plasma insulin concentration and the rate of insulin production.

THE LEVELS OF INSULIN IN MAN.

Following the introduction of the sensitive radio-immunoassay techniques, many reports appeared of the levels of insulin in health and disease, and useful hypotheses were derived years before the observed plasma insulin concentrations were shown to have a direct physiological relationship to insulin production rate and to its peripheral action on muscle. The recent identification of "proinsulin" and "big" insulin cast some doubt on the absolute specificity of the immunological methods, and biological activity of the measured insulin cannot always be assumed, although all available evidence indicates that only the secretion of pancreatic beta cells is assayed by these techniques.

In non-diabetic subjects during glucose tolerance tests, a very wide range of plasma insulin levels was reported at each sampling time, (Berson and Yalow 1960; Hales and Randle 1963) although for any individual the pattern of insulin response tended to be "in phase" with the blood glucose levels, showing a parallel rise and fall (Samols 1964). In these early reports, the non-diabetic subjects were not strictly categorized in terms of degree of obesity and level of health.

A large group of maturity-onset diabetic patients (Berson and Yalow 1960) showed a similar broad range of plasma insulin overlapping considerably with the non-diabetic subjects. Nevertheless, the characteristic pattern of response was of a delayed, sluggish rise of insulin values, eventually reaching excessive levels at two hours after oral glucose. This phenomenon was confirmed in adult diabetics of moderate severity (Hales and Randle 1963) but very mild adult diabetics showed a rapid and "appropriate" serum-insulin response exceeding that of normal subjects at 30 minutes after glucose.

The enigma of the delayed insulin secretory response of mild adult diabetics was further explored (Berson and Yalow 1961). Thus repeated oral glucose loading in normal subjects produced a plateau of insulin concentrations, with slightly declining blood sugar levels; whereas the diabetics showed a vast and progressive augmentation of plasma insulin reaching three times the mean normal level at 3 hours. Clearly the early diabetic, despite sluggish beta cells, has more than adequate reserves of immunochemical insulin, and the inability to maintain normal blood-sugar levels must result from failure of the secreted insulin to act as well as from the delayed secretory response.

Obesity was found to be associated with distinctly high levels of circulating insulin (Karam et alii 1963) whether or not diabetes was present. Since acromegalic patients, having a known antagonist to insulin action, showed a similar trait, the concept of insulin resistance being a primary aetiological event in the diabetes of obesity was promoted and it was suggested that hyperinsulinism represented a pre-diabetic phenomenon.

Juvenile diabetics almost invariably have shown low levels of serum insulin (Berson and Yalow 1962, Hales 1964) even in mild cases (Johansen and Lundbaek 1967) or during remission (Johansen and Ørskov 1969). The delayed rise of

insulin levels following glucose loading, characteristic of the maturity-onset diabetic with normal or elevated levels, is also seen in the insulin-deficient juvenile diabetics. The low insulin response appears to be genetically determined, for the monozygotic twin siblings of diabetics showed a close similarity in insulin levels during glucose infusion tests, whether or not impaired glucose tolerance was present (Cerasi and Luft, 1967). Similar results were obtained during oral glucose tolerance tests in the unaffected identical twins of diabetics (Pyke and Taylor 1967) and in the unaffected first-degree relatives of diabetics (Taylor et alii 1967) although some of the latter group, who developed symptoms of hypoglycaemia, exhibited transiently elevated insulin levels. Using the sustained glucose infusion technique in healthy subjects, Cerasi and Luft (1967) found that one-fifth of the group showed a low initial increment of plasma insulin and a low peak response and it was suggested that these subjects could be designated "potential diabetics" because of their identical pattern of response to the frank diabetics. The "biochemical inertia" of the pancreatic beta cell in all grades of adult diabetes, in comparison with normal and obese controls, (Seltzer et alii 1967) gave further emphasis to the importance of impaired insulin secretory response in the pathogenesis of hyperglycaemia.

THE VARIABILITY OF INSULIN LEVELS

It was to be hoped that the remarkable sensitivity, and high specificity, of the immunoassay would yield much useful information about the aetiology of diabetes. Indeed, in retrospect, it can be said that a number of the findings from bioassay studies have been substantially clarified. The presence of normal or high levels of circulating insulin in groups of mild maturity-onset diabetics has been confirmed; and the additional tendency for diabetics to show a delayed insulin

secretion after glucose stimulus has been established. But insulin values in the various diagnostic categories show a very wide range, with much scatter and overlap between groups. This finding perplexed clinicians and research workers alike, and aroused occasional scepticism about the validity of serum-insulin levels measured by immunoassay. Berson expressed the problem succinctly in the 1965 Banting Memorial Lecture:-

"The great biological variation in insulin-secretory responses suggests that significant differences in insulin sensitivity exist even among nonobese individuals without impairment of glucose tolerance, and that detailed analysis of a large series might conceivably provide some clues to aetiological factors determining insulin sensitivity"

It seemed important, therefore, to identify, if possible, factors relating to the variability of insulin levels encountered in health and disease. The identification of physiological associations would both confirm the "biological validity" of serum-insulin values, and would also assist in their interpretation. This objective was pursued in the studies to be reported in this thesis.

THE AETIOLOGY OF DIABETES

Diabetes mellitus remains a disease of unknown aetiology. Attempts to discern underlying mechanisms, perhaps the prime motive of much research into insulin measurement, has revealed useful but indirect clues. Thus "insulin resistance", inferred from the normal or high levels found in many cases of diabetes, has led to various searches for humoral antagonists of insulin action. Elevated levels of circulating free fatty acids have been proposed as a cause of insulin insensitivity, from the results of detailed 'in vitro' observations of animal tissues (Randle et alii 1963). In human subjects, carbohydrate intolerance, with high levels of circulating insulin and free fatty acids often coexist (Hales and Randle 1963). But whether

increased free fatty acid levels are the cause, or the effect, of "insulin resistance" has not been clearly proved, and requires further study, particularly in man (Ruderman et alii 1969). Other humoral factors invoked to account for insulin resistance are the synalbumin insulin antagonist of Vallance-Owen, for which no parallel studies of actual insulin levels have been made; and the chronic oversecretion of growth hormone (Young 1964). Although the latter possibility has been disfavoured on the basis of accurate measurements of growth hormone (Berson et alii 1965), interest in the pituitary origin of diabetes may be revived by recent reports of two polypeptide derivatives of growth hormone, one synergistic, and the other antagonistic to insulin's action, that appear to have biological significance (Armstrong et alii 1969; Bornstein et alii 1969).

The impaired secretory response of beta cells appears to be the hall-mark of severe diabetes, whether of the juvenile-onset type having marked insulin deficiency, or of adult-onset cases with at least normal levels of insulin. This trait appears to be hereditary in relatives of juvenile diabetics, at least, but presumably interacts with acquired factors. Although the measurement of insulin levels is not likely to provide evidence for the cause of defective insulin secretion, the relative contribution of this phenomenon and of "insulin resistance" require more clear definition in subjects with diabetes. This topic, therefore, was also pursued in the studies to be presented.

INSULIN AND VASCULAR DISEASE

It is common clinical experience that diabetics develop severe vascular complications, and at a younger age than the general population. Investigation of this problem led to the identification of early vascular changes in mild chemical diabetics, or in normoglycaemic subjects said to have "potential"

diabetes on the basis of a strong family history of the condition. In such subjects, abnormal conjunctival capillaries (Ditzel et alii 1954), diffuse glomerulo-sclerosis (Ellenburg 1962) and glomerular basement membrane thickening (Camerini-Davalos et alii 1963) were described. More recently, precise measurements of muscle capillary basement membranes have revealed that diabetics, and "potential" diabetics have a marked increase in the width of this structure (Siperstein et alii 1968), although confirmation of this finding is perhaps required. Nevertheless the concept that an anatomical lesion precedes the overt metabolic abnormalities of diabetes is intriguing. Impaired filtration of insulin from the vascular compartment of diabetic subjects had been proposed (Butterfield et alii 1963); thus it seems possible that the permeability of small blood vessels may dictate the rapidity of the metabolic response to insulin.

On the other hand, it is equally possible that the metabolic derangements of diabetes contribute to, or accelerate, vascular disease. The increased frequency of impaired glucose tolerance in patients with atherosclerotic vascular disease had been emphasized, both in clinical studies (Waddell and Field 1960; Sowton 1962) and in population surveys (Keen et alii 1965; Ostrander et alii 1965). Elevated blood-sugar levels were shown to be associated with coronary heart disease independently of serum-cholesterol and blood-pressure levels (Epstein et alii 1965). However, a metabolic factor operating in atherosclerosis other than hyperglycaemia (but common to diabetes) was suggested by the finding of increased synalbumin insulin antagonism in non-diabetic subjects with myocardial infarction (Vallance-Owen and Ashton 1963). Considerable interest was therefore aroused in the possible role of insulin in atherosclerosis, particularly when Mahler (1965) stated that the endothelial tissue of blood vessels responded to insulin, like

adipose tissue, by converting glucose to lipid, and by shutting down lipolysis. While assessment of insulin levels in patients with hypertension or peripheral vascular disease were in progress for the current thesis, elevated insulin values in normoglycaemic subjects with myocardial infarction were reported by Peters and Hales (1965). It seems very likely, therefore, that high insulin levels would prove to be associated with various manifestations of vascular disease, as an early phenomenon preceding abnormal glucose tolerance. Further studies were clearly important, for proof of such an association, while providing yet another "biochemical marker" for this common disease, would also suggest areas for future research into the aetiology of atherosclerosis. Insulin, conceivably, may have a direct role in the initiation of atherosclerotic lesions.

This completes the review of the literature and of recent developments relating to circulating insulin in health and disease.

The aims of the present study are presented on the next page.

THE MAJOR AIM of the present study was essentially: to determine factors related to the wide range of insulin levels encountered in man, in health and disease. In particular, the following objectives were pursued:-

- (a) definition of the relationship between serum-insulin and the corresponding blood-sugar levels, to discern the effectiveness of insulin secretion and insulin action;
- (b) definition of the relationships between serum-insulin and age, obesity, plus other physical and biochemical factors conceivably contributing to variability of insulin levels;
- (c) in diabetes, to clarify, if possible, the dual roles of "insulin resistance" and impaired insulin secretion in the pathogenesis of hyperglycaemia;
- (d) in vascular disease, to identify the mechanism of carbohydrate intolerance, and if possible, to acquire supporting evidence for insulin's aetiological role in atherosclerosis.

To achieve these aims, there had first to be developed a reliable and convenient technique for the immuno-assay of insulin.

CHAPTER 2

THE DOUBLE ANTIBODY IMMUNOASSAY OF INSULIN: ASSESSMENT AND MODIFICATION OF A METHOD.

INTRODUCTION

Yalow and Berson (1960) first presented a definitive immunoassay procedure for measurement of insulin after an extensive analysis of various factors influencing the reaction of insulin antisera with insulin, and of some sources of artefact in the separation of "antibody-bound" from "free" hormone by chromato-electrophoresis (Berson and Yalow, 1956, 1959). The relative simplicity of precipitating antigen-antibody complexes with appropriate antiglobulin sera had been shown by Skom and Talmage (1958) in their studies of acquired insulin-binding antibodies in human plasma, and this principle was applied to the immunoassay of insulin by Hales and Randle (1963) and Morgan and Lazarow (1963). Both methods made use of rabbit anti-guinea pig globulin antiserum (RAGPS) to precipitate the guinea-pig anti-insulin antibody (GPAIS).

Hales and Randle produced antibodies in rabbits to a crude guinea pig gamma-globulin obtained by ammonium sulphate precipitation. When this was added to a mixture of dilute GPAIS, with labelled and unlabelled insulin (i.e. constituents of the first antibody reaction) the GPAIS-insulin complexes could be separated from uncomplexed insulin by microfiltration through cellulose acetate membranes. Human plasma, however, prevented the expected recovery of antibody-bound insulin- I^{131} and a cross-reactivity between human and guinea-pig gamma-globulins was

observed. Pre-precipitation of the GPAIS by RAGPS therefore was employed since it was found that the pre-precipitated GPAIS could still react with the insulin in solution but the interfering effect of human proteins appeared to be abolished.

Morgan and Lazarow, using a rabbit antiserum to guinea pig whole serum, overcame the necessity for microfiltration of each assay sample by adding "carrier" whole guinea-pig serum (GPS) in relative excess to the first antibody reactants, followed by sufficient RAGPS to precipitate all the GPS (usually 0.1 ml. of 1:100 dilution) including the GPAIS (usually 0.1 ml. of 1:10,000 dilution). The visible precipitate so formed could be separated by centrifuging and decanting the supernatant solution. These workers observed that undiluted rat plasma in the assay system produced less than the expected proportion of insulin- I^{131} in the precipitates. The "inhibitor" in plasma acted on the precipitating reaction alone, and could be overcome by dilution of the plasma or by heating plasma to 56°C (Morgan, Sorenson and Lazarow, 1964A). It was shown later to have properties identical with complement (Morgan, Sorenson and Lazarow, 1964B).

The problems posed by the possible cross-reacting proteins or inhibitors of human serum or plasma were acute since impaired precipitation of GPAIS-insulin complexes yielded spuriously high insulin values. Although a number of reports indicated that RAGPS when added in excess enabled complete precipitation of the complexes (Hales and Randle, 1963; Morgan and Lazarow 1963; Kuyzuza and Samols 1964), it was found that both antigen (GPS) and antibody (RAGPS) excess caused sub-optimal precipitation (Samols and Bilkus 1964). The lack of certainty that absolute separation of antibody-bound from free hormone consistently occurred was the major disadvantage of the double antibody methods.

In this study the method of Morgan and Lazarow was evaluated and experiments were made to identify and overcome the sources of

artefact. This technique was selected because of its greater simplicity and convenience.

MATERIALS AND METHODS:

Dilution Medium. All dilutions of serum and reagents were made with borate buffer containing 8.25g. boric acid, 2.70g sodium hydroxide, 2g. bovine serum albumin fraction V (Sigma Company) and 100 mg. sodium thiomersalate; with glass distilled water and 11.6N hydrochloric acid (about 3ml.) added to make the total volume 1 litre and final pH 8.4 to 8.6.

Standard Insulin. Twice recrystallized human insulin, reference ELF/201263, of potency 23.25 international units per mg., (determined chromatographically by the method of Fenton, 1959) was donated by the Medical Research Council (Wellcome Laboratories). 21.7 μ g of this preparation was dissolved in 0.1ml of 3mM hydrochloric acid and was made up to 252 ml with the standard borate buffer containing 0.2% bovine albumin to yield a final concentration of 2000 μ U per ml. Aliquots of this solution were stored at -20°C until needed.

Guinea-pig anti-insulin sera (GPAIS). Initially, insulin antisera were produced in guinea-pigs by the method of Robinson and Wright (1961), using pure crystalline beef or pork insulin. Suitable antisera were selected on the basis of their titre when reacted with insulin- I^{131} , and also the slope of the standard curves obtained, and these antisera were used for the preliminary experiments evaluating the method. Subsequently, a commercial GPAIS, Burroughs Wellcome guinea pig anti-ox insulin serum, batch K5549, was used for all assays involving the measurement of serum samples. This is conveniently presented as a lyophilized powder and reconstitution to 8.0 ml. yields a concentration of GPAIS of 1:16,000. The performance of this antiserum has been excellent

Insulin-I¹³¹ and Insulin-I¹²⁵ were obtained from the Radiochemical Centre, Amersham. The iodinated insulin is prepared from purified crystalline bovine insulin of potency 24.3 I.U. per mg. Each vial contains 0.1 µg of iodinated insulin in 1 ml of phosphate buffer pH7.4 with 0.5% bovine serum albumin. Insulin-I¹²⁵ currently manufactured can be kept at 4°C for at least 4 weeks until use.

Guinea-pig serum (GPS). 5 to 7 ml. of blood is obtained from adult guinea pigs by cardiac puncture. The serum obtained is pooled, and stored in aliquots at -20°C.

Guinea-pig gamma globulin (GPγG). Whole serum obtained from guinea pigs was dialyzed overnight against 0.015M Sorenson's phosphate buffer pH 7.0. Any precipitate was discarded and 10 to 20 ml. of the clear supernatant applied to DEAE-Sephadex A50 (Pharmacia) that had been soaked in 2 to 3 changes of the same buffer and packed in a 30x3 cm. column. The protein-containing eluate, checked by ultraviolet spectroscopy, was concentrated in Visking tubing with Carbowax. Aliquots were submitted for cellulose acetate electrophoresis in a protein laboratory (Dr J.R.Hobbs, Postgraduate Medical School) for assessment of purity and for approximate quantitation. Only those batches of GPγG giving a single band on electrophoresis (corresponding to the gamma-globulin band of whole GPS tested simultaneously) were used for labelling or for producing rabbit anti-guinea pig gamma-globulin sera.

Labelled guinea-pig gamma globulin (GPγG-I¹³¹). An estimated 20 µg of pure GPγG was labelled by the method of Hunter and Greenwood (1962) and the protein peak of radioactivity purified by passage through a small DEAE-Sephadex A50 column (20 x 1.5 cm.) prepared as described in the above paragraph. The GPγG-I¹³¹ obtained, was checked by immunoelectrophoresis and autoradiography, and all detectable radioactivity conformed exactly to the arc of the guinea-pig gamma-globulin of whole GPS, thus establishing its purity.

Rabbit anti-guinea pig antiserum (RAGPS) and rabbit anti-guinea pig gamma-globulin (RAGPyG). RAGPS for the initial evaluation of the double antibody method was prepared using injections of GPS with Freund's adjuvant as described by Morgan and Lazarow, except that the injections were given subcutaneously. For the preparation of RAGPyG, GPyG was prepared from a pool of 60 ml. of serum from mycobacteria-inoculated guinea pigs, yielding approximately 200 mg. of the pure protein. Alequots of GPyG were homogenized with Freund's complete adjuvant immediately prior to injection, and 5-10 mg. GPyG were injected into each of a batch of rabbits using 4 subcutaneous areas drained by axillary and inguinal lymphatic systems. Seven to twenty days after the last of 3 monthly injections the animals were bled 40 ml. by ear vein and sera were assessed for "titre". Suitable sera were pooled, divided into alequots, and deep frozen. The antibody production of rabbits producing a good titre was generally maintained by monthly injections of GPyG plus adjuvant.

The double antibody immunoassay of Morgan and Lazarow. The following flow sheet illustrates the method used. The only major modification employed at this stage was to exclude the precipitate washing stage described by the authors.

1.0 ml.unlabelled insulin (standard or assay sample)	
+ 0.1 ml.insulin I ¹³¹ (6μU)	
+ 0.1 ml.GPAIS	(1:10,000)
MIX	
"First antibody reaction" at 4°C for 24 hours	
+ 0.1 ml.GPS	(1:100)
+ 0.1 ml.RAGPS	(1:4)
MIX	
"Second antibody reaction" at 4°C for 1 hour	
Centrifuge at 2000 r.p.m. for 20 minutes (4°C)	
Decant supernatant	
Count I ¹³¹ of precipitate and supernatant tubes	
in gamma well counter.	

Radioactive iodine was counted in a manual or automatic well counter, and background counts were subtracted from the counts obtained for each tube. The final assay metameter was then expressed as "percentage insulin-I¹³¹ bound", that is: -

$$\frac{(\text{Counts in precipitate}) \times 100}{(\text{Counts in precipitate}) + (\text{Counts in supernatant})} \%$$

Expression of the metameter in this way obviates the need to correct for radioactive decay, and in addition, the calculation of "total counts" can provide a quality control check on the accuracy of various procedures in the assay from pipetting through to the final counting stage.

RESULTS

EVALUATION OF THE RECOMMENDED TECHNIQUE:

Satisfactory standard curves were repeatedly obtained with bovine insulin standards. However, when serum samples obtained from non-diabetic subjects were assayed at serial dilutions down to 1:20, two patterns of results were obtained. Half of the sera gave comparatively consistent results over the entire range of dilution, and the apparent insulin levels measured were within the range expected from reports of other workers, whereas sera from the remaining subjects yielded widely variable results with apparent insulin values that were suspiciously high particularly in the less diluted samples.

Since human sera may contain cross-reacting proteins or inhibitors that impair the precipitation of GPS by RAGPS, the effect of adding RAGPS in increasing quantities in the second antibody reaction was assessed. In most of these experiments, a

large excess of RAGPS resulted in a further fall in the quantity of insulin- I^{131} precipitated, and the quantity of RAGPS required to obtain maximum precipitation of insulin- I^{131} varied with each serum sample. The addition of excess RAGPS therefore provided no solution to the problem of sub-optimal precipitation of antibody-bound insulin- I^{131} .

CROSS-REACTION BETWEEN HUMAN SERA AND "RAGPS".

Immunoelectrophoretic studies (performed by Dr J.R.Hobbs) showed that the crude RAGPS gave precipitin lines against all components of GPS but also against the alpha-2 and beta globulin fractions of 2 out of 3 human sera tested. Such cross-reactions seemed to be unlikely cause for the marked impairment of guinea pig gamma-globulin precipitation observed in the trial assays, but one effect appeared to be the production of considerably more bulky precipitates with certain human sera. The larger precipitates could conceivably result in the trapping or non-specific binding of "free" insulin- I^{131} , and the crude RAGPS was therefore discarded. When the more pure rabbit anti-guinea pig gamma-globulin (RAGPyG) serum was prepared, immunoelectrophoresis against GPS showed a single dense precipitin band corresponding to guinea pig gamma-globulin; and although a slight cross-reaction with human gamma-globulin was demonstrated, this proved to be of no significance in the eventual performance of the assay.

STUDIES OF THE SECOND ANTIBODY REACTION.

Labelled guinea pig gamma-globulin (GPYG- I^{131}) was prepared because of the need to measure directly the completeness of precipitation of GPyG in the second antibody reaction. The purity and immunological integrity of the GPyG- I^{131} was established by immunoelectrophoresis and autoradiography. GPyG- I^{131} was used in tracer concentration to monitor the precipitation reaction as follows:

1.0 ml. (standard diluent or the stated dilution of human serum or plasma)
+ 0.1 ml. GP γ G-I ¹³¹ (tracer concentration)
+ 0.1 ml. "carrier GP γ G (at concentration stated)
+ 0.1 ml. RAGP γ G (usually 1:4)
MIX
Reaction at 4°C for 4 to 24 hours (as stated)
Centrifuge; decant supernatant; count precipitate and supernatant tubes

The results are expressed as "percentage GP γ G-I¹³¹ precipitated".

The effect of human serum on the completeness of GP γ G-I¹³¹ precipitation was studied by incubating RAGP γ G with a range of concentrations of GP γ G in the presence and absence of human serum (diluted 1:10) for 18 hours as shown in Figure 1. In buffer solutions, a comparatively broad range of GP γ G concentrations produced maximal precipitation of GP γ G-I¹³¹; but the small addition of human serum caused a marked impairment of precipitation and the range of concentration of GP γ G at which peak precipitation occurred was much more narrow. The experiment described provided the basis for the subsequent standardization of each batch of second antibody reagents obtained. The interfering effect of human serum on the completeness of precipitation of GP γ G-I¹³¹ is a constant phenomenon.

Time of incubation and the effect of heparin on the second antibody reaction were evaluated in experiments designed to compare the rate of precipitation of GP γ G-I¹³¹ in the presence of serum and of heparinized plasma taken from the same blood samples. The results of one experiment are shown in

Figure 2 - on this occasion the "optimal" precipitation of GP γ G-I¹³¹ in buffer solution was 95%. It is seen that in tubes containing the serum dilution (1:10) the substantial interference with precipitation was evident at 4 hours incubation; and although increasing the duration of the reaction to 72 hours produced a marked increase in the proportion of GP γ G-I¹³¹ precipitated, the peak precipitation (88% at the "optimal" concentration of carrier GP γ G) was still less complete than in buffer solutions. This was confirmed in several tests. The tubes containing the heparinized plasma dilution (1:10) showed remarkable acceleration of the precipitation reaction, such that at 4 hours "optimal" levels of GP γ G-I¹³¹ precipitation were achieved over a broad range of concentrations of "carrier" GP γ G. At 72 hours' incubation the optimal precipitation was maintained but over an even greater range of concentrations of the GP γ G.

The desirable concentration of heparin required to overcome the inhibitory effect of serum was assessed in the experiments summarized in Table 1, which used optimal concentrations of GP γ G and RAGP γ G. While heparin had no discernable influence on the precipitation of GP γ G-I¹³¹ in buffer tubes, in the tubes containing serum (1:10), concentrations of heparin from 10 i.u. to 1000 i.u. per tube abolished the inhibitory effects of serum after 16 to 24 hours' incubation but not at 3 hours.

OPTIMAL CONDITIONS FOR THE PRECIPITATION REACTION.

In subsequent experiments and assays, the concentrations of GP γ G and RAGP γ G for maximum precipitation of GP γ G-I¹³¹ in the presence of serum were defined in standardization experiments similar to those depicted in Figures 1 and 2. The reaction was incubated for 16 to 24 hours, and 30 i.u. of heparin was added to each tube with one of the second antibody

reagents. In practice, the use of pure GPyG was found to be unnecessary, and batches of whole guinea pig serum (GPS) were standardized in a similar manner against RAGPyG.

STUDIES OF FIRST AND SECOND ANTIBODY REACTIONS.

Having defined suitable conditions for the effective separation of "free" from antibody-bound insulin, various factors likely to influence the entire assay procedure were studied by following the percentage insulin- I^{131} recovered in the final precipitate.

The bovine serum albumin (fraction V, Sigma) used in the standard diluent to prevent insulin- I^{131} sticking to glassware (Berson and Yalow 1960) was assayed for insulin content, which was found to be negligible, amounting to 0.3 μ U bovine insulin per gram of albumin.

The effect of time of incubation of the first antibody reaction was assessed using various anti-insulin sera at concentrations selected to give a sensitive standard curve for the assay of human serum samples. It was found that to achieve equilibrium a minimum incubation of 4 days was necessary for the more dilute standard insulin solutions and 5 days for serum samples diluted 1:10 and 1:5 (figure 3)

Species specificity of the commercial GPAIS (K5549) that was selected eventually for assay purposes was assessed. The GPAIS was reacted with equivalent concentrations of human and bovine insulin. The results in figure 4 indicated that, although the standard curves obtained were very similar, the GPAIS had an increased affinity for bovine insulin of sufficient magnitude to cause substantial under-estimation of human insulin concentrations if bovine insulin standards were used for reference. The necessity to use human standard insulin for assays of human sera was thereby emphasized.

Omission of washing the final precipitate appeared to be desirable if large numbers of samples were to be handled. The extent of non-specific co-precipitation of free insulin-I¹³¹ was therefore measured by incubating control tubes for the assay procedure in the GPAIS omitted. Table 2 shows that the binding of free insulin-I¹³¹ in the precipitate tubes remained constant over the range of serum volumes employed and was unlikely to be a source of variability in the assay.

MODIFICATIONS TO THE ASSAY.

Of the changes to the method developed by Morgan and Lazarow, the most important have been directed towards achieving complete and constant precipitation of GPAIS. Thus: -

- (1) Preparation of a more pure precipitating antiserum, RAGPyG, overcame any substantial cross-reaction with human serum proteins.
- (2) Careful standardization of the second antibody reactants was required to define the optimal concentrations of GPS and RAGPyG to be used. In the standardization procedure, the presence of human serum and the absence of heparin, and a short incubation period (4 to 8 hours) were necessary to produce an identifiable peak precipitation over a narrow range of GPS dilutions.
- (3) Addition of heparin in standard amounts with the second antibody reagents; and -
- (4) Prolonging the duration of the second antibody reaction to 24 hours, abolished the sub-optimal precipitation that previously occurred in the presence of human serum.

Further modifications suggested by the experimental findings were to extend the incubation of the first antibody reaction to 6 days so that equilibrium conditions were achieved, and to omit washing the precipitate. Flow-sheet for a typical assay was therefore: -

1.0 ml. unlabelled insulin (standard human insulin, or unknown serum diluted 1:10)
+ 0.1 ml. insulin-I ¹³¹ (3 μ U)
+ 0.1 ml. GPAIS (1:16,000)
MIX
"First antibody reaction" at 4°C for 6 days
+ 0.1 ml. GPS (1:100)
+ 0.1 ml. RAGP γ G (1:4) including 30 I.U. heparin
MIX
"Second antibody reaction" at 4°C for 24 hours.
Centrifuge at 3000 r.p.m. for 20 minutes (4°C)
Decant supernatant
Count I ¹³¹ in precipitate and supernatant tubes.

SPECIFICITY OF THE ASSAY.

Conventional tests for the specificity of immunoassays were applied to this double antibody technique. The principle underlying these procedures is that substances having immunological cross-reaction with, but chemical distinction from,

human insulin will show differing levels of competition with insulin-I¹³¹ for binding by the specific antibody. Non-parallel standard curves are thereby obtained. The validity of this principle has been illustrated by its use in discriminating satisfactorily the small species differences of insulin preparations (Berson and Yalow 1959). If human sera contain substances immunologically similar to, but chemically distinct from, crystalline human insulin, assaying a range of dilutions of sera should reveal consistent deviations of the estimated levels of insulin from the expected values. Table 3 gives the results obtained with dilutions of sera from 1.0 ml. to 0.1 ml, and from 0.1 ml. to 0.025 ml, and shows that, despite some variation in individual results, the anticipated concentrations in the diluted samples were obtained. Similarly, addition of standard human insulin of known amounts to various sera were proportionately accounted for by the assay, as shown in Table 4.

PERFORMANCE OF THE ASSAY.

The first 12 assays were performed using the modified assay procedure, and were employed for the measurement of some 600 unknown serum samples. All serum samples were estimated in duplicate, as were the standard insulin solutions except for the more dilute concentrations (0 μ U/ml to 5 μ U/ml) which were triplicate measurements. Sera were uniformly diluted 1:10. Sources of within -assay variability that were scrutinized in the first 3 assays included (1) the accuracy of duplicates - the mean difference in percentage insulin-I¹³¹ precipitated being a 1.6% for both standard insulin solutions and for serum samples; and (2) the reproducibility of total counts per sample - the coefficient of variation ranging from 1.4% to 2.1%. These trends of performance continued and represented negligible sources of error.

Standard curves for the 12 assays are detailed in Table 5, which gives the final assay metameters obtained, and are summarized in Figure 5, which shows the mean line from these values. The assay metameter, percentage insulin- I^{131} precipitated, is plotted on the Y-axis, and the standard insulin concentration on a logarithmic scale on the X-axis. The curves obtained have been consistently sigmoidal, although approaching linearity over the range of insulin concentrations 1.25 μ U/ml to 20 μ U/ml.

The sensitivity of the assay is difficult to ascertain quantitatively because of the curvi-linear nature of the standard curves. Comparing the mean of the triplicate values obtained for the 0 μ U/ml and the 1.25 μ U/ml standards, a significant difference (at the 5% level of probability) existed in each assay. Thus the absolute sensitivity can be stated as "less than 1.25 μ U/ml." In three of the assays, the sensitivity was "less than 0.625 μ U/ml" (Table 5). Since sera are diluted 1:10, however, the practical sensitivity is 10 times these values. Although it is common practice to interpolate values of unknown samples lying between zero and the lowest standard (see Yalow and Berson 1965), on this series of standard curves, there are reservations about the accuracy of readings beyond the linear portion of the curves, that is, for serum levels of insulin less than 12.5 μ U/ml. and greater than 200 μ U/ml.

A standard serum sample ("S") stored in deep frozen aliquots, was measured in each assay as a routine quality control procedure. The results obtained are shown in Table 6 and Figure 6. The mean value of 59.5 μ U/ml. with standard deviation 7.7 μ U/ml. yielded a coefficient of variation of 13.0%. There is the suggestion of a systematic drift towards higher values with the passage of time. The range of values obtained for percentage insulin- I^{131} precipitated did not exceed that of the standard insulin solutions (see Table 5 and Figure 5); and further examination of the observed levels of "S" and the slope of the

linear part of the standard curve, or the degree of combination of GPAIS with insulin-I¹³¹ in the buffer tubes, revealed no consistent relationship. No cause, therefore, was ascertained for the variability between assays of the serum standard.

Replicate determinations of unknown sera were analyzed to provide a further indication of the degree of between assay variability. The values obtained in 2 of various separate assays for 60 samples are shown in Figure 7. A reasonable reproducibility was achieved, and the linear correlation coefficient was 0.94. The extent of variability was of the same order as that obtained for the standard serum "S", as evident from the analysis in Table 6.

Efficiency of the precipitation reaction was followed in serum samples from the first 80 subjects studied. Fasting samples, which were likely to be more concentrated, were run with "monitor" tubes that contained the same constituents as assay tubes except that GP γ G-I¹³¹ was added instead of the insulin-I¹³¹, and the completeness of precipitation compared with that found in buffer solutions. As shown in Table 7, satisfactory precipitation of GP γ G-I¹³¹ occurred in the presence of all sera, and all individual samples were within 5% of the optimal precipitation found in buffer tubes.

DISCUSSION

Confidence in the double antibody immunoassay for insulin has been increased considerably by the adoption of modifications to overcome impaired precipitation of insulin-antibody complexes, as outlined on page 58. Morgan and Lazarow (1964B) suggested that complement in serum was the major cause of impaired precipitation. Heparin may therefore exert

its beneficial effect on immunoprecipitation through its anti-complement activity. Soeldner and Slone (1965) have also found that prolonging incubation and adding heparin to the second antibody reaction were necessary adaptations to the original method. Pre-precipitation techniques do not necessarily avoid the interfering effects of human serum (Sheldon and Taylor 1968, Brunfeld and Jorgenson 1967) and the addition of EDTA appears to be required to avoid obtaining spuriously high values. The need for careful standardization of the second antibody reagents is of equal importance, and must be emphasized. The major disadvantage of most immunoassay techniques other than chromatoelectrophoretic procedures, is that the essential separation of free from antibody-bound labelled hormone is not monitored for completeness. Nevertheless, the experience reported in this study of 80 consecutive sera extending over 7 assays indicates that complete precipitation of guinea pig gamma-globulin can regularly be obtained by employing the precautions and modifications described.

The reliability of the assay must be considered in terms of "within-assay" and "between-assay" variability. Within an assay, the sensitivity and the "precision" (i.e. negligible range of duplicate estimations combined with suitably steep slopes of standard curves) indicate a high degree of accuracy compared to bioassay procedures, as has been the experience of most other workers. Between assays, however, an unexpected degree of variability occurred when examining the repeated estimations of a single serum sample, or the difference between repeated measurements of unknown sera. When these assays were performed quality control data from other laboratories were not available for comparison or for the definition of limits of acceptability. Subsequently, a number of reports of various immunoassays have been published and the coefficients of variation for repeated estimates of

standard sera are shown in Table 8. Clearly, the variability encountered in the assay procedure of this study is representative of the experience of most immunoassay workers. Therefore, no retrospective "tolerance" limits were applied to the data and no assays were rejected. But in the results of the clinical studies that follow, careful consideration is given to the interpretation of results with reference to the quality control data.

The advantages of the double antibody assay of Morgan and Lazarow are essentially those of technical simplicity. Although the decanting of supernatants from precipitates is necessarily a manual procedure which is difficult to envisage automating, the method was considered more practical in our hands than chromato-electrophoresis, which requires elaborate equipment, and more simple than microfiltration procedures. The lack of commercially available precipitating reagents is perhaps a disadvantage, resulting in the necessity to maintain a colony of rabbits and guinea pigs to provide these substances. Recent techniques that promise to be more convenient are the "solid phase" assays of Catt et alii (1967), and the dextran-coated charcoal assay of Herbert (1965), and their evaluation over a period of time is awaited with interest.

SUMMARY

The double antibody immunoassay for insulin was evaluated and modifications were devised to overcome the interfering effects of human sera. A rabbit antibody to pure guinea pig gamma globulin was produced to minimize cross-reaction with human proteins. Labelled guinea-pig gamma globulin was used to study the completeness of the second antibody reaction and to standardize precisely the concentrations of reactants required for "optimal"

precipitation. Prolonging incubation of the second antibody reaction to 24 hours, and the addition of heparin were found to be necessary for complete separation of antibody-bound from free Insulin-I¹³¹. The first antibody reaction required 6 days incubation to achieve equilibrium in the presence of serum. The assay's sensitivity was less than 12.5 μ U per ml. for sera assayed at 0.1ml volumes. Dilution of sera and addition of human insulin gave proportionate recoveries. The coefficient of variation for a standard serum estimated in 12 assays was 13.0% which corresponds to the variability between assays reported by other workers. The assay technique has the advantage of technical simplicity.

TABLE 1

Completeness of the second antibody reaction: effect of serum with and without added heparin. A minimum amount of 10 - 50 i.u. per tube avoids incomplete precipitation in the presence of serum after 16 - 24 hours. (The optimal concentrations of GP γ G and RAGP γ G were used).

TIME OF INCUBATION	PERCENT OF GP γ G-I ¹³¹ PRECIPITATED*							
	1.0 ml buffer plus heparin -				1.0 ml serum dilution (1:10) plus heparin -			
	0 iu	10iu	50iu	1000iu	0iu	10iu	50iu	1000iu
(1) 3hours:	96%	-	96%	-	24%	85%	89%	-
(2) 16hours:	96%	98%	99%	100%	77%	98%	99%	99%
(3) 24hours:	100%	-	99%	-	93%	97%	98%	-

*Expressed as a percentage of the optimal % GP γ G-I¹³¹ precipitated for each batch of GP γ G-I¹³¹ used, which was 95% for experiments (1) and (3) and 83% for experiment (2).

TABLE 2

Minimal and consistent non-specific
 "co-precipitation" of Insulin-I¹³¹
 in control tubes set up without GPAIS.
 Note that this precipitation remains
 low in all tubes.

Percentage of Insulin-I¹³¹ precipitated in control tubes.

<u>Buffer</u>	<u>0.1 ml serum</u>	<u>0.5 ml serum</u>	<u>1.0 ml serum</u>
2.7% (N = 4)	2.7% (N = 5)	3.5% (N = 3)	3.5% (N = 3)

TABLE 3. Specificity of the assay: dilution of serum (in final sample volume of 1 ml) yields proportional concentrations of insulin.

SERUM SAMPLES	ASSAYED INSULIN CONCENTRATION (μ U/ml) IN SERUM VOLUMES: -				
	1.0ml	0.5ml	0.1ml	0.05ml	0.025ml
1.	13	11	8	-	-
2.	21	15	19	-	-
3.	23	27	26	-	-
4.	37	28	34	-	-
5.	34	40	43	-	-
6.	-	-	44	44	52
7.	-	-	62	60	74
8.	-	-	190	156	176

TABLE 4. Specificity of the assay: addition of human insulin gives quantitative recovery. (sample volumes 0.1 ml)

SERUM SAMPLES	INITIAL SERUM LEVEL	ADDED HUMAN INSULIN	EXPECTED LEVEL	OBSERVED LEVEL
	(μ U/ml):	(μ U):	(μ U/ml):	(μ U/ml):
1.	.18	+22	100	93
	.18	+5.5	83.5	84
2.	30	+22	52	60
	30	+5.5	35.5	39
3.	22	+22	44	48
	22	+5.5	27.5	30

TABLE 5. Summary of results obtained for standard curves and the standard serum ("S") in 12 assays.

STANDARD CURVES										STANDARD SERUM	
ASSAY NUMBER	ASSAY METAMETER (PERCENT INSULIN-I ¹³¹ PRECIPITATED) FOR HUMAN INSULIN CONCENTRATIONS (μU/ml.)								SENSI- TIVITY (μU/ml)	ASSAY META- METER (AND ESTIMATED CONCENTRA- TION) (μU/ml)	
	0	0.63	1.25	2.5	5.0	10.0	20.0	40.0			
1	63.1%	..	55.3%	47.6%	41.2%	31.6%	22.7%	16.7%	<1.25	40.0 (.54)	
2	64.7%	..	57.1%	49.1%	42.7%	32.9%	24.4%	-	<1.25	40.1 (.53)	
3	67.1%	..	62.1%	56.9%	48.0%	38.4%	27.5%	18.7%	<1.25	36.1 (.57)	
4	64.6%	60.6%	57.9%	47.4%	37.6%	26.0%	19.3%	-	<0.63	36.6 (.52)	
5	78.6%	74.5%	70.7%	61.1%	48.9%	35.8%	24.8%	17.0%	<0.63	46.3 (.58)	
6	40.5%	33.4%	29.3%	27.7%	21.6%	15.9%	11.1%	8.0%	<0.63	22.1 (.62)	
7	40.2%	36.7%	33.1%	28.4%	21.7%	16.7%	11.1%	9.1%	<1.25	19.7 (.66)	
8	73.3%	-	65.2%	57.4%	45.4%	33.1%	23.4%	-	<1.25	39.4 (.70)	
9	67.5%	-	59.9%	53.5%	44.5%	37.0%	33.2%	27.2%	<1.25	43.1 (.57)	
10	71.1%	68.9%	63.6%	56.2%	45.1%	34.4%	25.5%	18.4%	<1.25	39.1 (.66)	
11	4340*	4278*	4016*	3548*	2768*	2110*	1642*	1228*	<1.25	3080 (.47)	
12	59.9%	55.6%	53.5%	48.2%	41.0%	37.1%	32.0%	-	<1.25	38.9 (.66)	
Means:	63.1%	...	55.5%	48.5%	39.7%	30.2%	22.3%	16.4%	...	36.3% (60)	

* precipitates only counted.

TABLE 6.

Summary of results of replicate determinations of unknown sera between assays. The estimate of the standard deviation is derived from $\sqrt{\Sigma^2/2N}$, where d = difference between replicates, and N = number of samples.

RANGE OF CONCENTRATION	ESTIMATE OF STANDARD DEVIATION	NUMBER OF SAMPLES.
5 - 50 μ U/ml	6.7	(43)
51 - 100 μ U/ml	10.2	(14)
101 - 190 μ U/ml	27.7	(4)

TABLE 7. Completeness of precipitation of tracer amounts of labelled guinea pig gamma globulin (GP γ -I¹³¹) added to control tubes in 7 assays. For 80 consecutive serum samples, precipitation of GP γ -I¹³¹ was optimal, i.e. always within 5% of the precipitation obtained in buffer tubes.

<u>ASSAY NUMBER</u>	<u>PERCENTAGE OF GPγ-I¹³¹ PRECIPITATED IN MONITOR TUBES</u>			
	<u>Serum Samples (1:10)</u>			
	<u>Buffer</u>	<u>Mean</u>	<u>Range</u>	<u>Number Tested</u>
1:	90%	88%	86-90%	(3)
2:	83%	84%	82-87%	(15)
3:	80%	80%	77-83%	(13)
4:	87%	85%	83-86%	(4)
5:	81%	80%	79-81%	(12)
6:	88%	86%	85-88%	(20)
7:	81%	80%	79-81%	(14)

TABLE 8 Coefficients of variation for repeated estimates of standard sera "between assay" in various immunoassay procedures reported.

INVESTIGATORS	IMMUNOASSAY: Hormone & Method	COEFFICIENT OF VARIATION
Boden & Soeldner (1967)	HGH (double antibody)	14.3%
Buchanan & McKiddie (1968)	Insulin (double antibody)	(16.7% (18.7%
Catt et al.(1967)	HGH (solid phase)	(6.8% (17.3%
Catt et al.(1969)	Angiotensin 11 (charcoal)	12 %
Fraser & Wright (1968)	HGH (double antibody)	(14-17% (23-25%
Garcia (1967)	HGH (double antibody)	10 %
Mitchell (1969)	HGH (enzyme partition)	21.1 %
Rodbard et al.(1968)	LH (double antibody)	13.2 %

HGH = human growth hormone

LH = lutenizing hormone

0.1 ml. RAGP γ ($\frac{1}{2}$)
 0.1 ml. "carrier" GP γ G
 at concentration shown
 0.1 ml. GP γ G-I¹³¹ (trace)
 1.0 ml. buffer or serum
 at 1:10 dilution

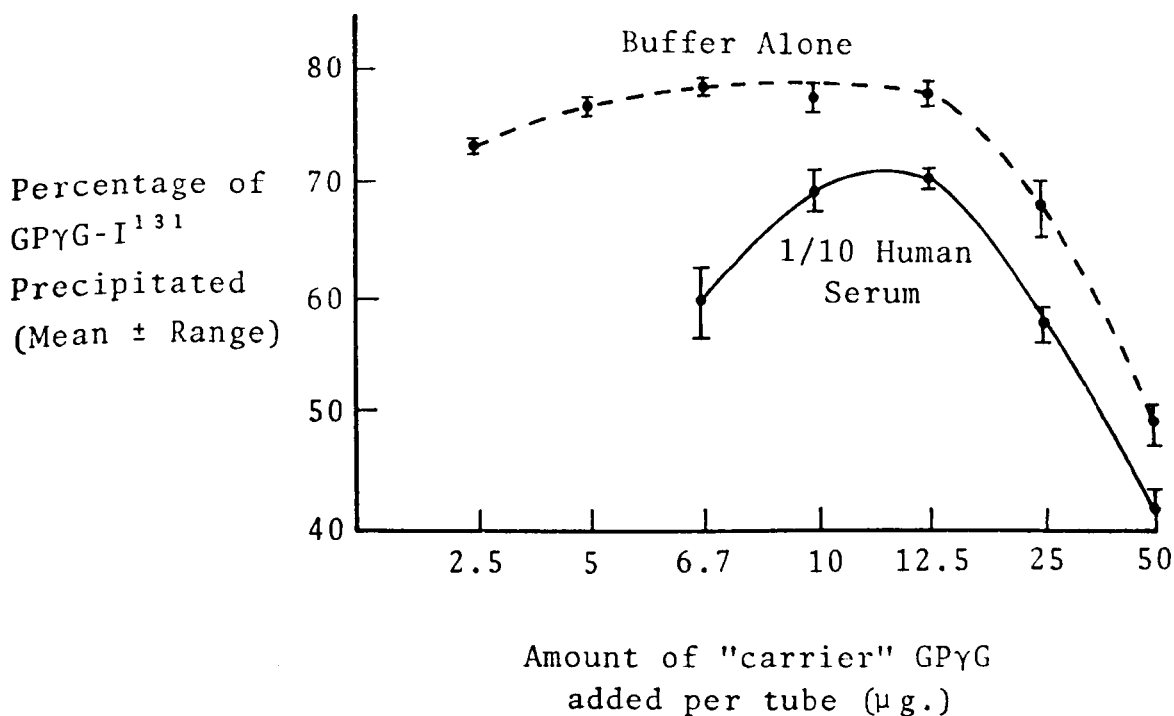


FIGURE 1. Completeness of the second antibody reaction in buffer solutions, and the effect of human serum (0.1 ml.). An 18 hour incubation was employed.

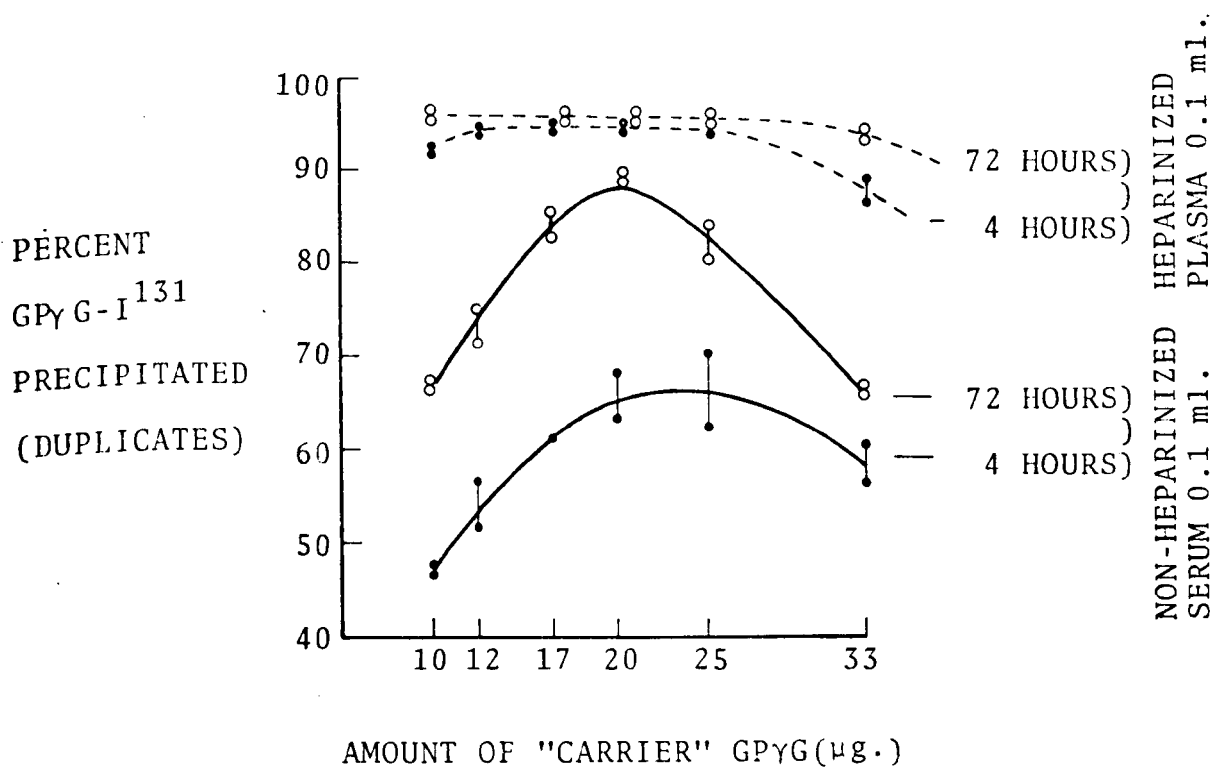


FIGURE 2.

Completeness of the second antibody reaction in tubes containing serum (0.1 ml) compared with plasma (0.1 ml) and the effect of time of incubation.

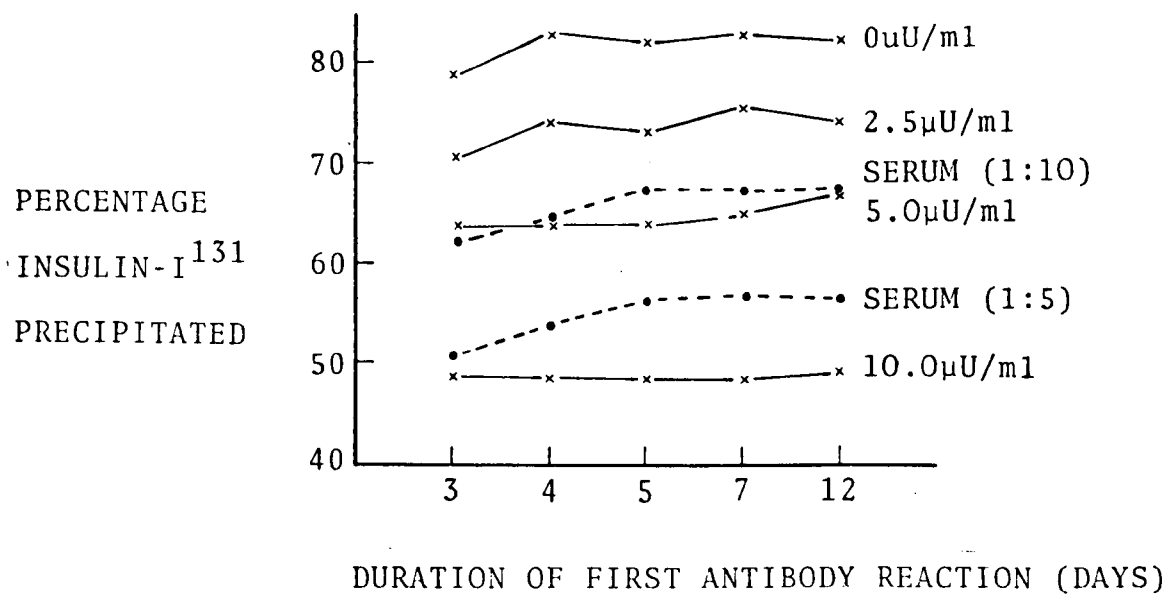
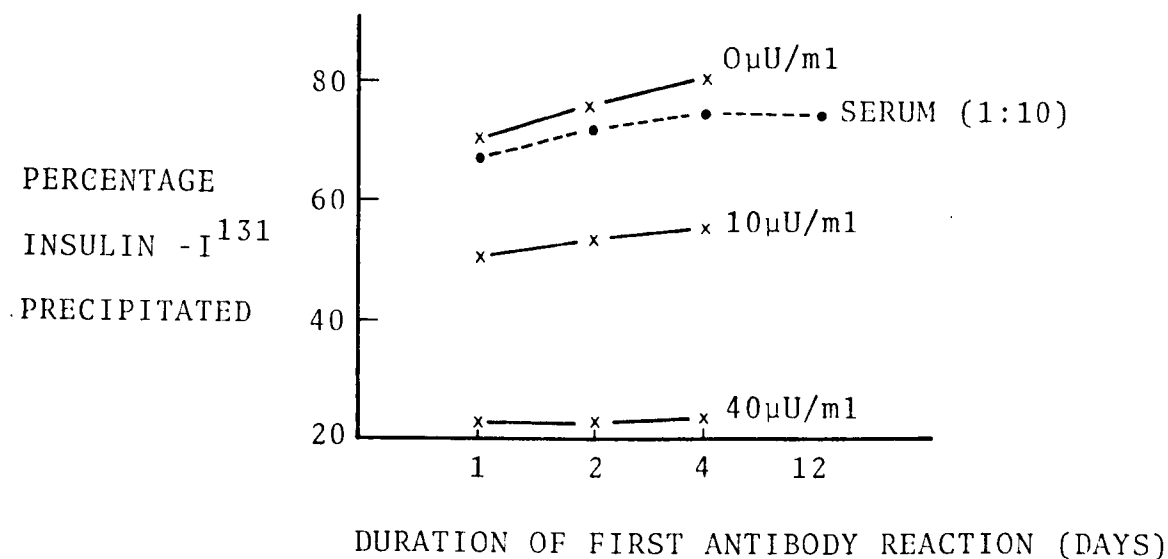


FIGURE 3.

Effect of time of incubation of first antibody reaction for standard insulin solutions and for serum samples (diluted 1:5 and 1:10). 5 days is taken to achieve equilibrium.

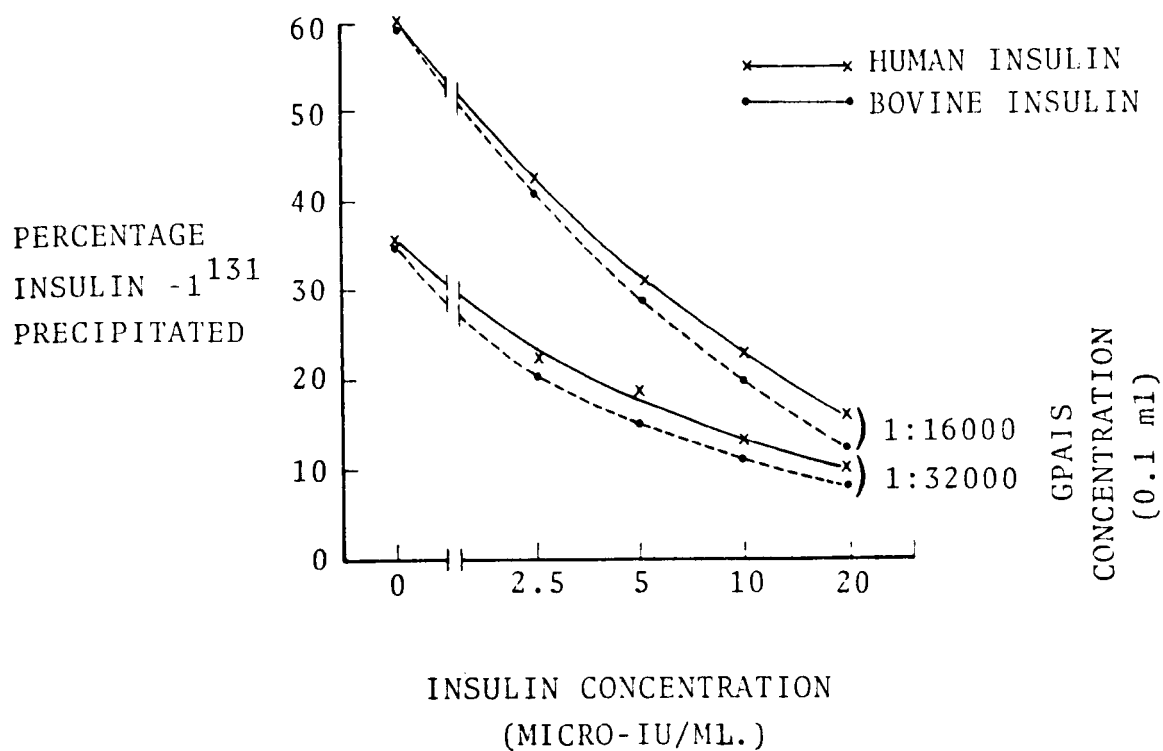


FIGURE 4.

Species specificity of guinea pig anti-insulin serum (K 5549). The GPAIS had greater affinity for bovine than for human insulin.

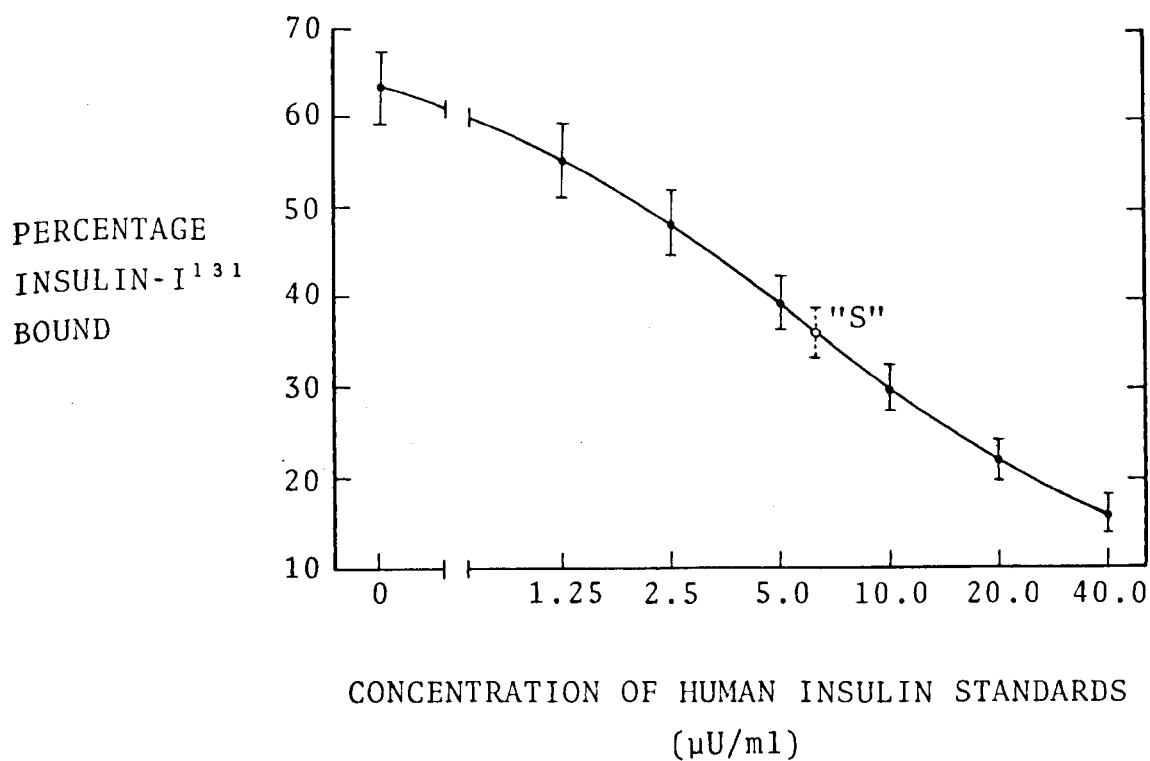


FIGURE 5.

Mean values (± 1 standard error) of the assay metameters obtained for standard curves in 12 assays. The concentration of insulin standards is plotted on a logarithmic scale. "S" indicates the range of values obtained for the standard serum.

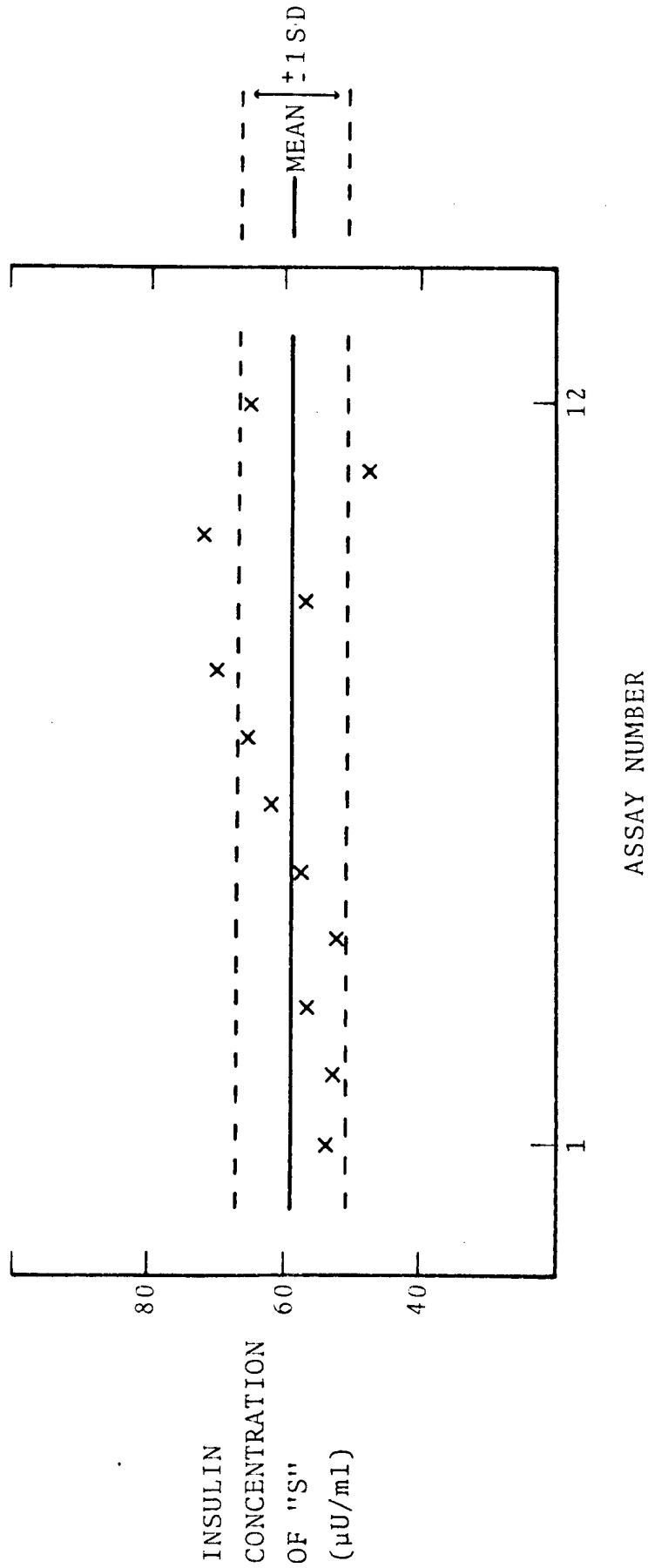


FIGURE 6.

Quality control chart: insulin values obtained for aliquots of standard serum "S" in each of 12 assays. The coefficient of variation was 13.0%.

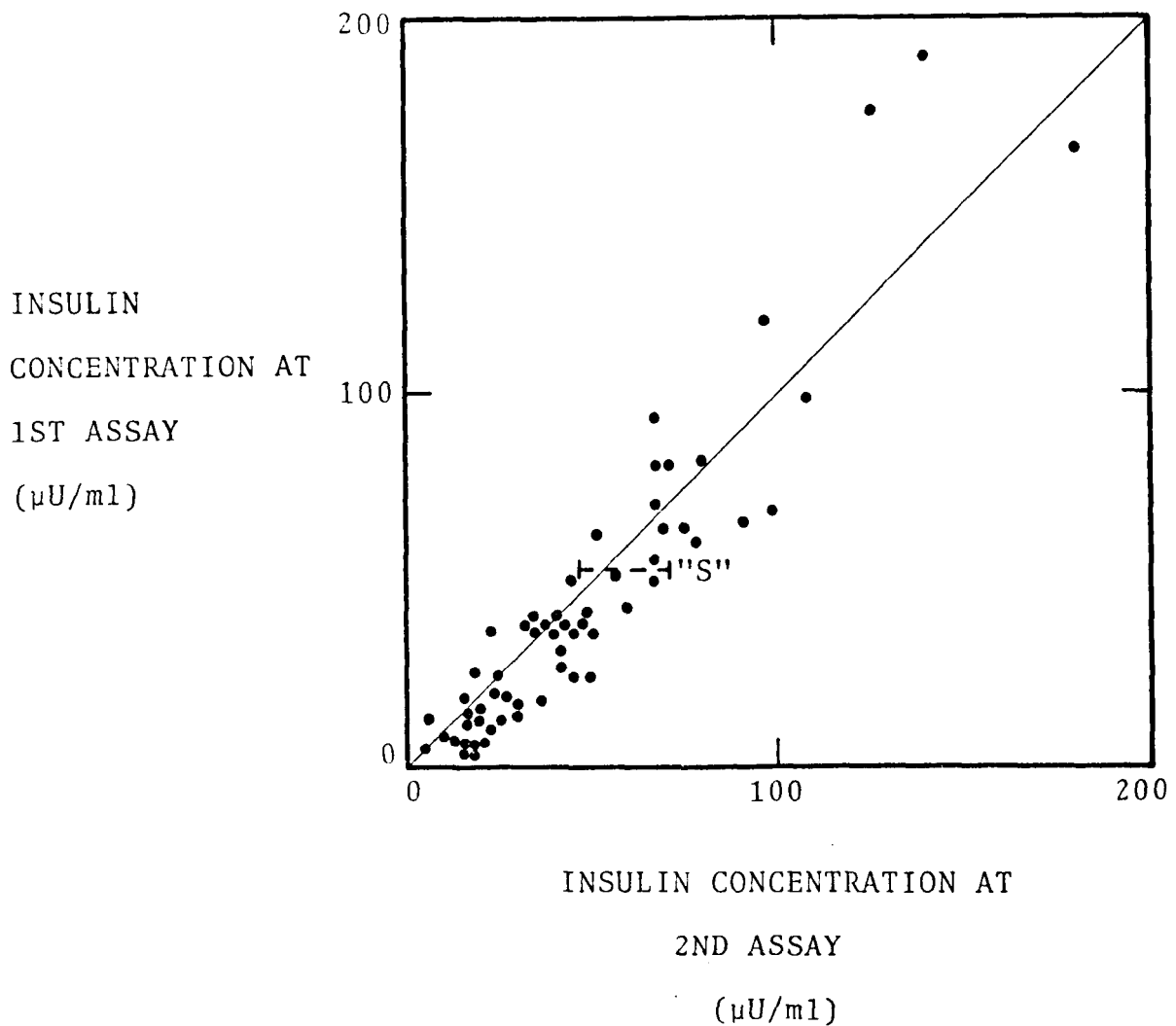


FIGURE 7.

Replicate determinations, between assay, of unknown serum samples. The correlation coefficient was 0.94.

CHAPTER 3

THE NORMAL INSULIN RESPONSE TO ORAL GLUCOSE

INTRODUCTION

A wide range of serum-insulin values in non-diabetic subjects had been reported by Yalow and Berson (1960) and by Hales and Randle (1963). The former study made use of 30 patients attending a general laboratory for glucose tolerance tests. The mean levels of serum-insulin (and the absolute range of values) were 20 $\mu\text{U/ml}$ (0-66 $\mu\text{U/ml}$) basally; and 139 $\mu\text{U/ml}$ (18 to 342 $\mu\text{U/ml}$) at one hour after 100 g. oral glucose. The latter report concerned 5 selected normal men; the means and ranges are given later in Table 12. In order to explore in some greater detail the insulin response to oral glucose, and to provide a working normal range for comparison with diseased subjects, a larger group of healthy volunteers was selected, by more stringent criteria, for study.

METHODS

SELECTION OF VOLUNTEERS.

Members of the hospital staff and some London University students were chosen on the initial basis of stated good health and the absence of any known metabolic disorder. Retrospective criteria employed for inclusion in the study were (1) a normal oral glucose tolerance test (GTT) result by Hammersmith Hospital criteria (i.e. venous blood-sugar less than 100 mg/100 ml in the fasting state, and less than 120 mg/100 ml at 120 minutes after glucose using "true glucose" methods; and no complications to the test);

(2) body weight not exceeding 115% of the expected mean for age and height (Kemsley 1951); and (3) "White European" race. Of 50 volunteers tested, 5 were then excluded on the grounds of nausea or faintness during the test (2 subjects), excessive body weight (1 subject) and different racial origin (2 subjects, one of whom was also found to be a subclinical diabetic). Four volunteers had a family history of diabetes in parents and/or siblings, and 4 of vascular diseases (i.e. coronary heart disease, claudication or hypertension) but were not excluded on these grounds. Age was not a specific criterion of selection; the mean age for 22 males was 30 (range 23-47) years, and for 23 females 29 (range 18-52) years.

TEST PROCEDURE.

Volunteers were asked to ensure a reasonable carbohydrate intake for 3 days before testing. After an overnight fast of 10 - 14 hours, with subjects remaining seated or at rest in bed for the duration of the test, a solution of 50 g. of dextrose monohydrate in approximately 200 ml. of water was taken orally within 5 minutes. Venous blood samples were obtained fasting and at 30, 60 and 120 minutes after glucose in all cases, and in the last 22 cases, venous and "arterialized" capillary blood samples were obtained concurrently at 45 minutes also. In most cases the subcutaneous fat-fold thickness of the mid-triceps area was measured with Harpenden calipers in the recommended manner (Tanner 1959). Blood-sugar was measured by the ferricyanide method (Hoffman 1937) on the Autoanalyzer, which gives values very close to true glucose.

INSULIN ASSAY : QUALITY CONTROL.

The samples were estimated in assays 1 to 7 (Chapter 2) in which the mean value for the standard serum "S" was 58.5 μ U/ml and the standard deviation 4.5. It was observed that high and low insulin secretors did not emerge from any particular assay day.

STATISTICAL METHODS.

The "integrated insulin response" on the "insulin area" was calculated for the 2 hours of the GTT from the formula

$$\text{Insulin area} = \frac{a + 2b + 3c + 2d}{4}$$

where a = fasting; b = 30 min., c = 60 min; d = 120 min. serum-insulin levels.

This provides an index of the total insulin secretion during the GTT, and since it is expressed as a single figure, enables the ranking of subjects from lowest to highest insulin secretors.

Multiple regression analysis was performed using a standard program (Stenhouse 1965) as described in Appendix I.

RESULTS

Data obtained from the 22 males and the 23 females are shown in Table 1. The mean values for age, percentage desirable weight, and blood-sugar levels indicate that the sexes were well matched for these variables.

DISTRIBUTION AND RANGE OF INSULIN VALUES.

The distribution of insulin values obtained at each sampling time was observed to be positively skewed, except for the fasting values (Table 2A). Using the absolute insulin

levels, there was a discrepancy between means and medians; the standard deviations were large so that 2 standard deviations less than the mean gave negative values; and the distribution of serum-insulin values at 60 and 120 minutes differed significantly from the expected symmetrical normal distribution by the chi-squared test. Conversion of the serum-insulin levels to logarithms (to the base 10) resulted in a close correspondence of mean and median values and a distribution that tended to fit, and certainly did not differ significantly from, the expected normal distribution (Table 2B). Standard deviations of the $\log_{10}$ serum-insulin values were very similar at all sampling times. A useful normal range could now be calculated from the mean $\log_{10}$ levels $\pm$ 2 standard deviations (Table 3).

RELATION OF SERUM-INSULIN TO OTHER FACTORS.

The very wide scatter of the absolute values encountered led to analysis of factors possibly associated with this variability. A preliminary assessment of such factors was made by comparing the means of $\log_{10}$ serum-insulin in sub-groups with the mean values and range of $\pm$ 1 standard deviation of the whole group and is summarized in Figure 1. The results indicate that the variations of sex, age, degree of adiposity, and family history of diabetes or vascular disease, did not make any substantial contribution to the wide range of insulin levels observed.

EFFECT OF SEX.

Table 4 compares the mean $\log_{10}$ serum-insulin levels ($\pm$ 1 standard deviation) for the 22 males and the 23 females. No difference was found between male and female levels except for the mean fasting levels, where the difference, although just significant, was small and unimportant. For the remainder

of this presentation, therefore, data from both sexes has been pooled, since separate analyses, with the small numbers involved, did not lead to more useful conclusions.

RELATION TO BLOOD-SUGAR LEVELS.

In the course of obtaining assay results, it was observed that certain healthy volunteers showed consistently high serum-insulin levels and these "high insulin secretors" tended to differ from the remaining subjects by having a peak insulin level at 60 minutes rather than 30 minutes after glucose, and by exhibiting generally higher levels of blood-sugar. This phenomenon was pursued by calculating the "integrated insulin response" for the 45 subjects. The subjects were then ranked from the lowest to highest values, and the data was divided into quintiles (i.e. groups of 9 subjects). The mean $\log_{10}$ serum-insulin levels and the mean blood-sugar levels for these quintiles of insulin area are shown in Figure 2. It is evident that individuals tended to retain a characteristic tendency for higher or lower insulin levels throughout the GTT, at all sampling times. Also the lower 3 quintiles showed a maximum mean insulin level at 30 minutes, whereas the upper 2 quintiles' peak response was delayed by comparison, to 60 minutes. With regard to the mean blood-sugar levels of these groups, the lower 3 quintiles showed similarity and considerable overlap, but the 2 highest quintiles exhibited distinctly elevated mean blood-sugar levels at 30 and 60 minutes. However, at 120 minutes, the highest insulin secreting group, with the highest blood-sugars earlier in the GTT, tended to have relative hypoglycaemia (mean blood-sugar level 54 mg/100 ml).

SIMPLE CORRELATIONS OF BLOOD-SUGAR AND SERUM-INSULIN.

A correlation matrix of all variables measured in the 45 subjects revealed correlations of borderline significance between body weight and fasting $\log_{10}$ serum-insulin ($r = 0.32$, $P < 0.05$), sex and fasting $\log_{10}$ serum-insulin ($r = 0.33$, $P < 0.05$). Height, percentage desirable weight, mid-triceps fat-fold, or age had no significant correlations with any of the blood sugar or $\log_{10}$ serum-insulin levels obtained from this comparatively homogeneous group. Inter-relationships between blood-sugar levels and the $\log_{10}$ serum-insulin levels are shown in Table 5. The correlations between $\log_{10}$ serum-insulin at any one sampling time with the insulin values for other times were all positive, confirming the trend for high or low insulin secretors to remain so throughout the test. For blood-sugar and $\log_{10}$ serum-insulin relationships at the same time, the 60 minute levels ($r = 0.58$, $P < 0.001$) and the 120 minute levels ($r = 0.48$, $P < 0.001$) showed particularly strong correlations.

MULTIPLE REGRESSION ANALYSIS.

To clarify the independent relationships of variables correlating with serum insulin, multiple regression analysis was performed as follows:

<u>Dependent Variable.</u>	<u>Determining Variables.</u>
(1) $\log_{10}$ serum-insulin (at each sampling time)	(2) 0 min. blood sugar
	(3) 30 min. blood sugar
	(4) 60 min. blood sugar
	(5) 120 min. blood sugar
	(6) age
	(7) Sex (male = 1) (female = 2)
	(8) Height
	(9) Weight

The results in Table 6 show the three separate multiple regression equations for "prediction" of $\log_{10}$ serum-insulin levels at 0, 60 and 120 minutes respectively. Each analysis has excluded age, sex and the blood-sugar values at 0 and 30 minutes for having no significant independent contribution to the regression equation. (In the analysis of the 30 minutes $\log_{10}$ serum-insulin, not one of the determining variables contributed at a significant level, and therefore this is not shown.) In our sample, it is clear that the $\log_{10}$ serum-insulin levels at 0, 60 and 120 minutes each had significant independent associations with the blood-sugar level at 60 and/or 120 minutes, and in particular there was a highly significant association between the 60 minute $\log_{10}$ serum-insulin and the 60 minute blood-sugar ($P < 0.001$) and between the 120 minute $\log_{10}$ serum-insulin and the 120 minute blood-sugar ($P < 0.001$). The coefficient of multiple correlation was highest for the 60 minute $\log_{10}$ serum-insulin ($R = 0.6875$) and 45% ($A = 0.4476$) of its variability was accounted for by the positive relationship with the 60 minute blood-sugar and the negative relationship with the 120 minute blood-sugar. Weight was positively associated with the fasting $\log_{10}$ serum-insulin and height negatively with the 120 minute $\log_{10}$ serum-insulin but both determinants were of borderline significance.

The blood-sugar levels as the dependent variables were similarly analysed by multiple regression on the following basis:

<u>Dependent Variable</u>	<u>Determining Variables</u>
(1) Blood-sugar level (at each sampling time)	(2) 0 min. $\log_{10}$ serum-insulin (3) 30 min. $\log_{10}$ serum-insulin (4) 60 min. $\log_{10}$ serum-insulin (5) 120 min. $\log_{10}$ serum-insulin

The final regression equations in Table 7 will not be described in detail, but it is apparent that the insulin levels during GTT "determined" to a greater or lesser extent the blood-sugar levels at each time and about 50% of the variability of the 60 minute and 120 minute blood-sugar levels can be "predicted" by the insulin levels. It should be noted that the 60 minute $\log_{10}$ serum-insulin was a significant determining variable in each of the four equations.

CAPILLARY BLOOD-SUGAR LEVELS.

It was recognised that arterial blood-sugar levels would indicate more accurately the stimulus for insulin secretion, and also that insulin's effect would be discerned by arterio-venous differences in blood-sugar concentration. The data obtained from arterialized capillary blood-sugars collected simultaneously with venous blood 45 minutes after glucose in 22 subjects is summarized in Table 8. The correlation coefficients between the 45 minute $\log_{10}$ serum-insulin and the venous and capillary blood sugars, and the capillary-venous difference, may be positive but are not significant on this small sample. However, the coefficients for the latter two are greater and suggest that a relationship may emerge with larger numbers.

THE EFFECT OF DIET.

The volunteers had been taking their usual diet before the GTT (although all had been asked to ensure a reasonable carbohydrate intake in the preceeding 3 days). Subsequently, a dietitian assessed the diet over the past year of 7 available high insulin secretors and 6 low insulin secretors to provide Table 9; there was no striking difference in dietary pattern of the two groups.

THE "NORMAL RANGE" FOR SERUM-INSULIN.

A "normal range" may be calculated from the data by defining the 95% fiducial limits (mean $\pm$ 2 standard deviations) of the symmetrical log-normal distribution of serum insulin for each sampling time (see Table 3). However, because of the strong relationship of $\log_{10}$ serum-insulin with the blood-sugar, it appeared that taking the latter into account might give a more useful estimate of the "expected" serum-insulin. When the blood-sugar and $\log_{10}$ serum-insulin values from the 45 subjects at four sampling times (0, 30, 60 and 120 minutes) were pooled to give 180 paired observations, the following regression of $\log_{10}$ serum-insulin on blood-sugar was derived:

$$\log_{10} \text{ serum-insulin} = 0.574 + 0.903 \times (\text{blood sugar in mg/100 ml})$$

$$\text{Correlation coefficient (r)} = 0.601$$

Significance of regression

by student's "T" test: $t = 10.0$ ($P < 0.001$)

The fiducial limits of (1) the regression line (equivalent to the range of two standard errors from the mean), and of (2) any point on the line (equivalent to the range of two standard deviations from the mean) are shown in Figure 3 and summarized in Table 10. The average standard deviation for $\log_{10}$ serum-insulin, given a blood-sugar level, is 0.32 - somewhat larger than the standard deviations obtained at any one sampling time (Table 4). Also the Figure shows, for each of the sampling times, the mean point and 95% range of $\log_{10}$ serum-insulin and blood-sugar values. This indicates clearly that the fasting sample values are quite dissimilar from the remaining samples, as perhaps would be expected. Therefore, the range for the fasting serum-insulin levels were defined without regard to the blood-sugar levels as the fiducial limits of the mean, as derived from Table 2B and as shown in Figure 4.

The data from the 30, 60 and 120 minute samples were then pooled to give 135 paired observations and the following regression equation of $\log_{10}$ serum-insulin on blood-sugar was derived; -

$$\log_{10} \text{ serum-insulin} = 0.867 + 0.070 \times (\text{blood sugar in mg/100 ml})$$

$$\text{Correlation coefficient (r)} = 0.647$$

Significance of regression
by student's "T" test: $t = 9.8$ ($P < 0.001$)

No appreciable deviation from linearity emerges on plotting the individual points, which are symmetrically disposed about the regression line. The fiducial limits of the regression line and of a point on the line are shown in Figure 4 and in Table 11. The range of $\log_{10}$ serum-insulin, for a given blood-sugar, is narrowed and the mean points for 30, 60 and 120 minute samples fall close to the overall line. Furthermore, the average standard deviation for $\log_{10}$ serum-insulin, at a given blood-sugar level, is 0.24, which is equivalent to the standard deviations for any one sampling time (Table 4).

DISCUSSION

This normal group, while not representative of the general population, were selected from young intelligent volunteers who were accepted on the basis of known good health. The subjects studied were within fairly narrow limits of age and "desirable" body weight, and their glucose tolerances were normal. Selection of healthy young subjects may often give a better indication of the "ideal" range of a variable for the definition of "normality" than random sampling from the general population, which will give the prevailing range of values in a group having a variable departure from ideal health (McCall 1967). In any case, despite selection into a

narrowly defined group, the insulin levels showed a very wide range. Variability of the assay did not appear to contribute substantially to the scatter of values. The distribution of serum-insulin levels at each sampling time of the glucose tolerance test were unimodal but were positively skewed. Logarithmic conversion of the serum levels yielded a symmetrical and apparently normal distribution of values, a desirable property for the purposes of obtaining means and ranges, and for statistical comparisons.

In analysing the wide range of absolute insulin values encountered, it was observed that the tendency to have high (or low) insulin values was constant throughout the test, and that the subjects with higher values tended to have both a more delayed peak insulin level (at 60 minutes) and higher blood-sugar levels at 30 and 60 minutes. Although the 120 minute blood-sugar levels were not widely scattered, the quintile of highest insulin secretors showed a trend towards mild relative hypoglycaemia at this time. This finding was reminiscent of the observed characteristic of maturity-onset diabetics to show delayed hyperinsulinism in response to a glucose load (Berson and Yalow 1960) and sometimes to exhibit late hypoglycaemia (Seltzer, Fajans and Conn 1956).

Within this normal healthy group, therefore, small trends could be discerned that mirrored the more substantial abnormalities recognized in diabetes. In particular, the phenomenon of "insulin resistance" became apparent, namely the coexistence of high insulin levels with relatively high blood-sugar values. The data cannot indicate a cause for "insulin resistance" but the possible explanations are the same as those put forward to account for observations in maturity-onset diabetes: namely (1) the insulin secreted may have impaired biological activity ("abnormal" insulin or

"proinsulin"); (2) insulin may be bound by larger serum proteins or polymerized so that its egress from the vascular compartment is relatively delayed; (3) circulating insulin antagonists could impair insulin action on target tissues, particularly muscle, but also possibly liver, and adipose tissue; (4) differences in vascular permeability could account for such hyperinsulinism with hyperglycaemia; (5) target tissue sensitivity may differ from subject to subject.

The relationship of the wide range of insulin values to other measured variables was examined, using correlation and multiple regression analyses. The results indicated that in this group, age, sex, and parameters of body size had no substantial contribution to the range of $\log_{10}$ serum-insulin levels, although the independent associations of body weight with the fasting serum insulin, and height (negatively) with the 120 minute serum insulin, suggested that some definite relationships might emerge from a study of larger numbers of subjects. Separate comparisons of the few subjects with positive family histories of diabetes or vascular disease with the remainder of the group, and a dietary analysis of "high" versus "low" insulin secretors did not reveal that these factors were a source of variability.

The strong mutual relationship between blood-sugar levels and serum-insulin values emerged on correlation studies (Table 5) and was confirmed by multiple regression analyses (Tables 6 and 7). This provides evidence that the wide range of insulin values has a biological, rather than an artefactual, basis. The positive and highly significant association of corresponding blood-sugar and serum-insulin values at 60 minutes, and similarly at 120 minutes, gives a further clear indication that degrees of "insulin resistance"

exist in these subjects. Thus the conclusion from multiple regression analysis that association with blood-sugar levels accounts for a substantial proportion of the variability of serum-insulin, may be interpreted in a physiological sense as showing that "insulin resistance" contributes to the wide range of levels found even in healthy subjects.

The fasting and the 30 minute insulin values failed to correlate with their corresponding blood-sugar levels. The fasting insulin levels were the least accurately measured, and the fasting blood-sugar concentrations were within a narrow range. Also, it is evident from Figure 3 that the association between these fasting parameters, if any, differs from that obtaining after the glucose load. It is of course, a long-standing observation that glucose is a major stimulus for pancreatic insulin release (Anderson and Long 1947). In addition, the influence of the various possible "gut factors" (secretin, pancreozymin, intestinal glucagon) operate to augment insulin release when oral glucose is administered. At 30 minutes after glucose, variable rates of intestinal glucose absorption, and comparatively rapid changes in glucose and insulin levels, may have accounted for the failure of the 30 minute $\log_{10}$ serum-insulin to be related to any of the determining variables.

At 60 minutes after glucose, the best "prediction" of the $\log_{10}$ serum-insulin as the dependent variable was obtained by multiple regression analysis. Forty-five percent of the variability of these values was accounted for by the regression (Table 6). Also, when blood-sugar levels were analysed as dependent variables, at each of the four sampling times, there was a significant association with the 60 minute insulin value (Table 7). It seems likely that in healthy subjects at one hour glucose absorption was mostly complete,

and in the entire group, blood-sugar levels, while not necessarily "steady" were declining uniformly, thus the relationship between glucose and insulin in blood would be better defined. That a lesser proportion (29%) of the variability of the 120 minute log serum-insulin was determined by the 120 minute blood-sugar (and by height) may have reflected variable trends of blood-sugar and insulin, in that certain subjects may have achieved a hypoglycaemic trough before, and others after, this sampling time. Also, the influence of falling blood-sugar levels is likely to have stimulated growth hormone secretion (Roth et alii 1964). It would appear that the sampling time of one hour after 50 g. glucose gives a useful measure of insulin secretory response in normal subjects. A suitable indication of the peak insulin response is obtained, and the high insulin secretors are probably most clearly discriminated. These findings influenced the choice of the one hour sampling time in the population study to be described.

It is now the common experience of workers using the immunoassay that although valuable conclusions may be derived from comparison of group differences, the interpretation of individual levels is difficult. Essentially, this is due to the very wide range of values encountered. Expressing the range of insulin values for a given blood-sugar concentration had the theoretical virtue of relating insulin response to the "stimulus" (although ideally arterial blood samples should be used, but these are impractical). The 95% confidence limits for any serum-insulin level, given a venous blood-sugar level at 30, 60 or 120 minutes after glucose showed a range of values no greater than at any one sampling time in the group of healthy volunteers. The range defined (Figure 4) is of some clinical use in providing an index of suspicion in certain circumstances. For example, "insulin

resistance" may be inferred by finding high levels of insulin with normal or mildly elevated blood-sugar levels, as in obesity, acromegaly, or Cushing's syndrome (Karam et alii 1963). Conversely consistently low insulin values may suggest "genetic pre-diabetes" (Pyke and Taylor 1967; Cerasi and Luft 1967) particularly if the rise after glucose is sluggish (Seltzer et alii 1967). This graphical technique of relating the serum-insulin response to the blood-sugar levels has some similarity in principle to the "insulinogenic index" (Seltzer et alii 1967), which expresses as a ratio the "incremental insulin area" over the "incremental glucose area".

The wide range of insulin levels may merely reflect biological variation; but further studies were indicated to identify the relationships of serum-insulin with physiological variables other than the blood-sugar level, and to assess the significance of "high" or "low" insulin secretors in the normal population.

Comparison of the insulin levels obtained in this study with other recent reports of lean healthy subjects is shown in Table 12. Double antibody immunoassay methods were used, and with one exception (Soeldner and Slone 1965), suitable human insulin standards. The range of values obtained in the different studies is similar. Workers using post-precipitation assays (Soeldner and Slone 1965; Chlouverakis et alii 1967) have obtained low fasting values comparable to our own; whereas the pre-precipitation and microfiltration procedure (Hales and Randle 1963) used by the remaining workers tends to give generally higher values.

SUMMARY

The normal insulin response to oral glucose was studied under controlled conditions in 45 selected healthy volunteers, all of whom were lean and had normal glucose tolerance. Serum-insulin levels showed a wide (tenfold) range, and the distribution of values was shown to be log-normal. No consistent effect of sex, age, body weight, preceding diet, nor of family history of diabetes or vascular disease, was discerned. A strong association of insulin values with simultaneous blood-sugar levels was evident, particularly at 60 minutes after glucose. Thus the high insulin secretors tended to have higher blood-sugar levels: "insulin resistance" therefore accounts in part for the wide range of insulin values found. The high insulin secretors tended to remain so throughout the test, and showed a delayed peak insulin response. A normal range of serum-insulin levels has been defined for purposes of comparison with disease groups. In the assessment of serum-insulin levels during glucose tolerance tests, the blood-sugar levels may be usefully taken into account. Studies of further variables related to the wide range of insulin values are clearly indicated.

TABLE 1. Physical Measurements and Blood-Sugar and Serum-⁹⁶
 Insulin Concentrations during Glucose Tolerance
 Tests in 45 Healthy Volunteers.

SUBJECTS	AGE (years)	HEIGHT (inches)	WEIGHT (pounds)	% OF MEAN EX- PECTED WEIGHT %	MID-TRICEPS FAT-FOLD (mm)
(A) <u>22 MALES</u>					
1.	23	71	175	112	12
2.	23	64	117	89	7
3.	25	71	151	96	-
4.	27	71	169	107	14
5.	28	76	203	115	14
6.	28.	72	164	101	11
7.	28	72	161	100	14
8.	28	74	170	100	10
9.	28	70	151	97	6
10.	29	72	170	104	16
11.	29	74	175	103	12
12.	29	70	168	108	10
13.	29	72	154	94	6
14.	29	71	145	91	8
15.	30	73	176	106	-
16.	31	69	149	97	9
17.	31	72	171	104	10
18.	31	74	166	91	13
19.	33	67	140	95	-
20.	35	67	154	105	10
21.	37	71	180	111	10
22.	47	71	127	79	10
MEANS (± 1 SD)	30 $\pm$ 5	71 $\pm$ 3	161 $\pm$ 19	100 $\pm$ 9	11 $\pm$ 3

TABLE 1. (Cntd)

SUBJECTS	AGE (Years)	HEIGHT (inches)	WEIGHT (pounds)	% OF MEAN EX- PECTED WEIGHT %	MID-TRICEPS FAT-FOLD (mm)
(B) <u>23 FEMALES</u>					
1.	18	66	144	112	15
2.	21	67	140	105	17
3.	22	61	110	97	17
4.	23	67	126	95	14
5.	24	64	124	100	14
6.	24	66	132	102	17
7.	24	64	141	114	22
8.	24	66	136	105	19
9.	26	65	133	104	22
10.	26	67	140	104	20
11.	26	70	125	87	12
12.	27	64	132	95	13
13.	27	67	138	103	21
14.	30	67	125	93	12
15.	31	65	126	97	15
16.	31	65	118	91	14
17.	31	65	135	104	23
18.	32	62	117	97	-
19.	35	65	126	96	-
20.	36	63	123	97	16
21.	37	61	134	111	25
22.	37	66	120	88	18
23.	52	64	124	88	13
MEANS ($\pm 1SD$)	29 ± 6	68 ± 4	145 ± 22	99 ± 8	17 ± 4

TABLE 2 Serum-insulin levels in 45 healthy subjects during GTT. The mean values, standard deviations (SDs) and the distributions at the various sample times are shown for (A) Absolute serum-insulin levels and (B) $\log_{10}$ serum-insulin.

(A) ABSOLUTE SERUM -INSULIN LEVELS

Sample time (min.)	Serum-insulin (uU / ml)			No. of subjects in the range: -				χ^2 *
	Mean	Median	SD	less than - 1SD	mean to - 1SD	mean to + 1SD	greater than + 1SD	
0:	9.4	9	4.5	7	16	14	8	0.2 (p>0.95)
30:	49.8	42	27.4	4	22	12	7	5.1 (p=0.1-0.2)
45:	53.6	47	31.5	2	12	6	2	4.3 (p=0.2-0.3)
60:	48.1	35	30.2	2	26	9	8	13.9 (p<0.01)
120:	22.5	17	17.0	1	30	7	7	24.0 (p<0.001)

(B) $\log_{10}$ SERUM-INSULIN LEVELS

Sample time (min)	$\log_{10}$ serum-insulin			No. of subjects in the range: -				χ^2 *
	Mean	Median	SD	less than -1SD	mean to -1SD	mean to +1SD	greater than +1SD	
0:	0.94	0.95	0.24	6	22	10	7	5.0 (p=0.1-0.2)
30:	1.64	1.62	0.23	6	18	14	7	0.8 (p=0.8-0.9)
45:	1.66	1.67	0.24	4	7	7	4	0.3 (p>0.95)
60:	1.61	1.54	0.25	6	19	12	8	1.9 (p=0.5-0.6)
120:	1.26	1.23	0.26	8	15	15	7	0.1 (p>0.99)

* Comparison of observed distribution with the expected distribution for 45 subjects (7.2, 15.4, 15.4, 7.2) or for 22 subjects (3.5, 7.5, 7.5, 3.5) with 3 degrees of freedom.

TABLE 3. The normal range of Serum-Insulin values (converted from $\log_{10}$ serum insulin) for 45 healthy volunteers during 50 G oral GTT.

Sample time (minutes)	Mean (M)	Range ($M \pm 2SD$)
0 :	9 μ U/ml.	3-26 μ U/ml
30 :	44 μ U/ml.	15-125 "
45 :	47 "	16-137 "
60 :	41 "	13-131 "
120 :	18 "	6-60 "

TABLE 4. Comparison of $\log_{10}$ serum-insulin of 22 males and 23 females.

Sample time (minutes)	Mean log ₁₀ serum-insulin ([±] 1SD)				Significance
	22 males		23 females		
0 :	0.99 [±]	0.19(10μU/ml)	0.83 [±]	0.25(7 μU/ml)	(p<0.05)
30 :	1.68 [±]	0.24(48 ")	1.61 [±]	0.22(41 ")	(n.s. *)
60 :	1.61 [±]	0.28(41 ")	1.60 [±]	0.25(40 ")	(n.s.)
120 :	1.19 [±]	0.21(15 ")	1.31 [±]	0.29(20 ")	(n.s.)

* n.s. = not significant.

TABLE 5.

Correlation coefficients for blood sugar (mg/100ml) and $\log_{10}$ serum insulin at the various sampling times during GTT for 45 normal subjects. Non-significant coefficients are shown in parentheses, and the degree of significance of the remainder are indicated below the diagonal by asterisks.

Correlation Coefficients (r):

		Blood-sugar at time (min.) :				$\log_{10}$ Serum-insulin at time (min.) :			
		0	30	60	120	0	30	60	120
Blood sugar at time (min.):	0 :		(.28)	(.27)	(.14)	(-.24)	(-.17)	(.23)	(.07)
	30 :	-		.63	(-.09)	(-.08)	(.19)	.51	(-.03)
	60 :	-	***		(.06)	-.30	(-.07)	.58	(.09)
	120 :	-	-	-		-.37	-.31	-.34	.48
$\log_{10}$ serum- insulin at time (min.):	0 :	-	-	*	*		.50	(.19)	(.10)
	30 :	-	-	-	*	***		.49	(.24)
	60 :	-	***	***	*	-	***		(.15)
	120 :	-	-	-	***	-	-	-	

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

TABLE 6.

Multiple regression analysis of factors determining $\log_{10}$ serum-insulin at 0, 60, and 120 minutes in 45 healthy subjects. The final regression equations are read vertically. The significance of the contribution of each determining variable has been assessed by Students' t Test and is shown in parentheses.

REGRESSION EQUATIONS FOR DEPENDENT VARIABLES:				
	Serum- $\log_{10}$ Insulin	60min. $\log_{10}$ Serum- Insulin	120min $\log_{10}$ Serum- Insulin	
Y INTERCEPT - - - - -	0.970	1.327	2.363	
60 min. Blood sugar (mg/100ml) :	-0.299 $\times 10^{-2}$ (p<0.05)	+0.618 $\times 10^{-2}$ (p<0.001)	...	
120 min. Blood sugar (mg/100ml) :	-0.434 $\times 10^{-2}$ (p<0.05)	-0.496 $\times 10^{-2}$ (p<0.01)	+0.751 $\times 10^{-2}$ (p<0.001)	
Weight (pounds) :	+0.349 $\times 10^{-2}$ (p<0.05)	...	...	
Height (inches) :	...	...	-0.233 $\times 10^{-1}$ (p<0.05)	
Coefficient of Multiple Correlation ("R") :	0.552	0.688	0.574	
Proportion of Variability Ascribable to the Regression ("A") :	0.254	0.448	0.298	

TABLE 7.

Multiple Regression Analysis of factors determining blood-sugar levels at 0,30,60 and 120 minutes in 45 healthy subjects, with the corresponding $\log_{10}$ Serum-Insulin as determining variables. The data is set out in a comparable fashion to Table 6.

REGRESSION EQUATIONS FOR DEPENDENT VARIABLES:					
	0min. Blood Sugar	30min Blood Sugar	60min Blood Sugar	120min Blood Sugar	
Y INTERCEPT - - - - -	74.2	56.7	56.0	84.2	
PARTIAL REGRESSION COEFFICIENTS FOR DETERMIN- ING VARIABLES	Omin.log ₁₀ Serum-Insulin: ...	...	-28.76 (p<0.05)	-27.62 (p<0.01)	
	30min.log ₁₀ Serum-Insulin: -10.51 (p<0.05)	...	-33.82 (p<0.05)	...	
	60min.log ₁₀ Serum-Insulin: +10.12 (p<0.05)	+34.17 (p<0.001)	+75.96 (p<0.001)	-26.66 (p<0.01)	
	120min.log ₁₀ Serum-Insulin: ...	...	...	+37.79 (p<0.001)	
Coefficient of Multiple Correlation	0.397	0.509	0.750	0.729	
Proportion of Variability Ascribable to the Regression ("A"):	0.118	0.242	0.530	0.498	

TABLE 8. Correlation of the venous and capillary blood-sugar levels, and the capillary-venous difference in blood sugar, with $\log_{10}$ serum-insulin at 45 mins. after glucose.

22 Subjects : 45 min. blood sample		
	Mean $\pm$ 1S.D	Correlation coefficient with $\log_{10}$ serum-insulin
Venous blood sugar (V) :	$106 \pm 25 \text{mg}/100\text{ml}$	0.20 (N.S.*)
Capillary blood sugar (C):	$132 \pm 29 \text{mg}/100\text{ml}$	0.28 (N.S.)
(C) — (V):	$26 \pm 12 \text{mg}/100\text{ml}$	0.28 (N.S.)

+ N.S. = not significant.

TABLE 9.

Assessment of Diet (over preceding year) for 6 Low Insulin Secretors and 7 High Insulin Secretors.

	"Insulin area" (unit-hours, mean and range)	Surface area (sq. m., mean and range)	(Diet analysis (mean [±] S.D.))			
			Total calories (per sq.m.)	Carbohydrate (g. per sq.m.)	Protein (g. per sq.m.)	Fat (g. per sq. m.)
Low insulin secretors (6)	43 (35-51)	1.88 (1.62-2.24)	1368 ([±] 340)	125 ([±] 18)	49 ([±] 17)	71 ([±] 13)
High insulin secretors (7)	122 (102-172)	1.76 (1.46-1.97)	1373 ([±] 400)	140 ([±] 34)	48 ([±] 13)	68 ([±] 16)

TABLE 10. Regression of $\log_{10}$ Serum-Insulin on blood-sugar for 180 paired observations at 0,30, 60 and 120 minutes (45 subjects). The values are given for $\log_{10}$ Serum-Insulin and for the fiducial limits at the blood-sugar levels stated (see Figure 3.)

Log ₁₀ Serum-Insulin Levels at blood-sugar levels:			
	45mg/100ml.	100mg/100ml.	155mg/100ml.
Mean :	0.982	1.478	1.974
+2 S.E. :	+0.087	+0.052	+0.131
+2 S.D. :	+0.626	+0.623	+0.634
("Average" standard deviation = 0.32*)			

TABLE 11. Regression of $\log_{10}$ Serum-Insulin on blood-sugar for 135 paired observations at 30,60, and 120 minutes (45 subjects). The values are given for $\log_{10}$ Serum-Insulin and for the fiducial limits at the blood-sugar levels stated (see also Figure 4.)

Log ₁₀ Serum-Insulin Levels at blood-sugar levels:			
	45mg/100ml.	100mg/100ml.	155mg/100ml.
Mean :	1.184	1.568	1.952
+2 S.E. :	+0.076	+0.043	+0.100
+2 S.D. :	+0.475	+0.471	+0.479
("Average standard deviation = 0.24*)			

*Compare with Table 4.

TABLE 12. Serum-Insulin results obtained by various workers using double-antibody immunoassays in the study of lean healthy subjects during 50g. oral glucose tolerance tests.

Investigators	Mean (and range) of Serum-Insulin at sampling times:	
	0 mins.	60 mins
Hales and Randle (1963)*	16(6-25) μ U/ml.	65(43-120) μ U/ml.
Soeldner and Slone (1965)*	8.5(1-20) μ U/ml.	 μ U/ml.
PRESENT STUDY (1965)+	9(3-26) μ U/ml.	41(13-131) μ U/ml.
Chlouverakis et alii (1967)+	8(4-13) μ U/ml.	32(9-115) μ U/ml.
	10(4-26) μ U/ml.	43(12-160) μ U/ml.
Buchanan and McKiddie(1967)#	20(7-54) μ U/ml.	74(29-141) μ U/ml.
Jorgensen (1968)+	18(6-26) μ U/ml.	74(36-143) μ U/ml.
Hales et alii (1968)*	20(11-31) μ U/ml.	44(14-120) μ U/ml.

* Means and absolute range of arithmetic values.

+ Means and 95% range of $\log_{10}$ values.

Mean and 68% range of $\log_{10}$ values.

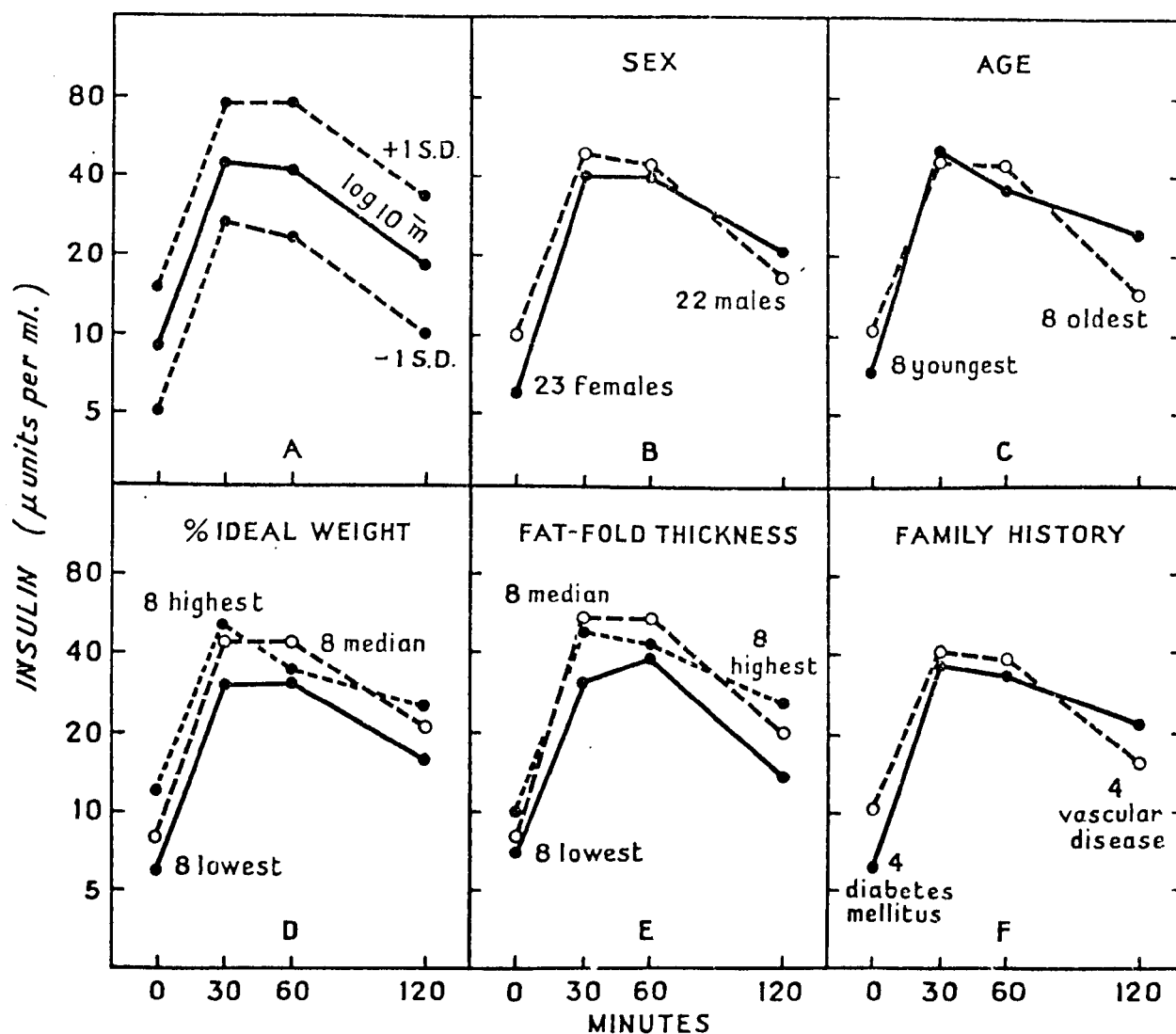


FIGURE 1.

Log₁₀ mean insulin curves for:

(A) whole group, with ± 1 S.D.: (B) 22 males versus 23 females; (C) 8 youngest and 8 oldest; (D) highest, median, and lowest % ideal weight (groups of 8); (E) highest, median, and lowest fat-fold thickness (groups of 8); (F) volunteers with family history of diabetes and of vascular disease (4 each). All groups show no appreciable deviation from the mean values for whole group.

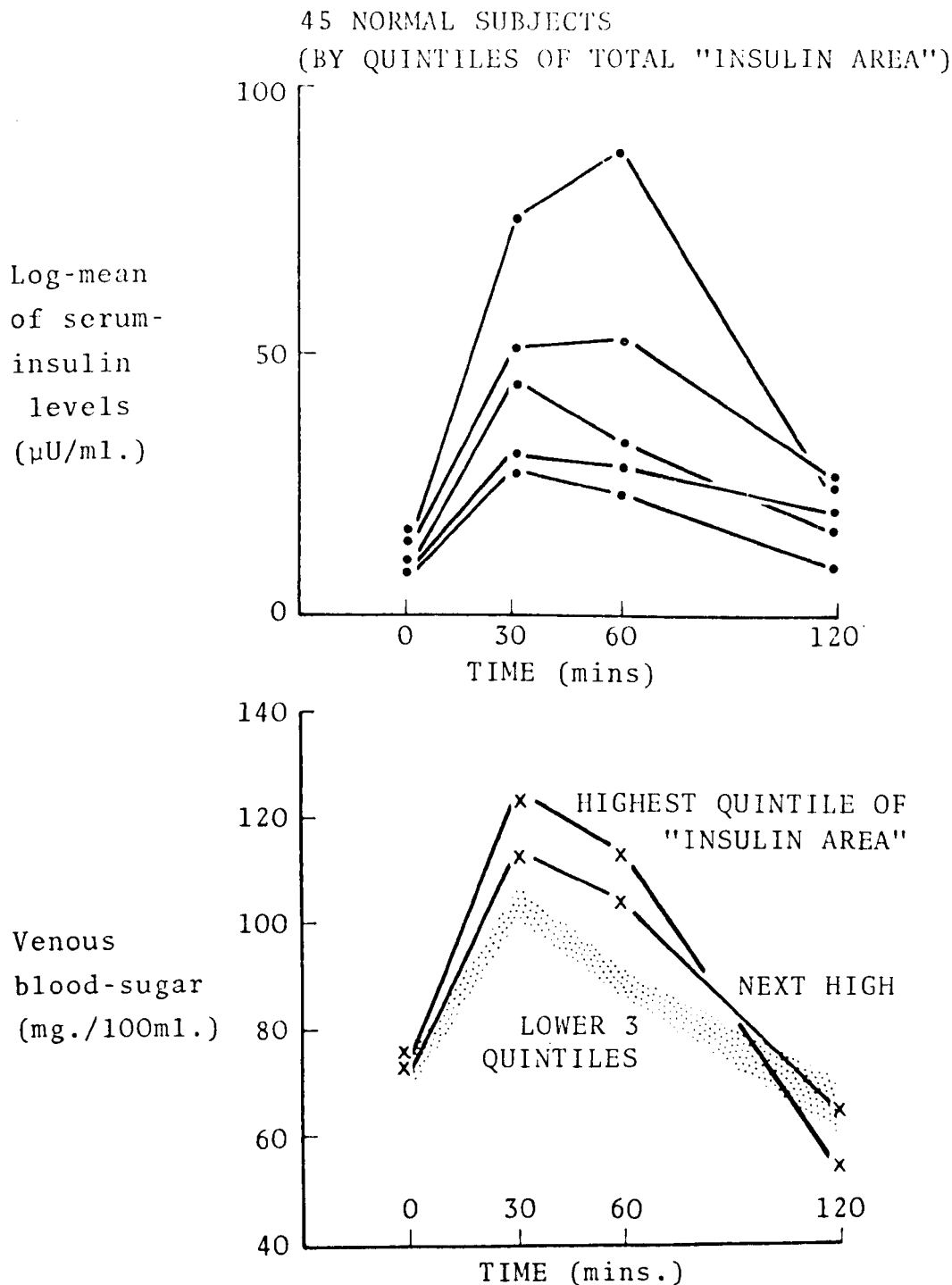


FIGURE 2.

Mean $\log_{10}$ serum-insulin values, and mean blood-sugar levels, for 5 groups of 9 volunteers ranked from lowest to highest insulin secretors by total insulin "area". Note consistent trend to high or low insulin levels at all sampling times; also the high insulin secretors tend to have higher blood-sugar values.

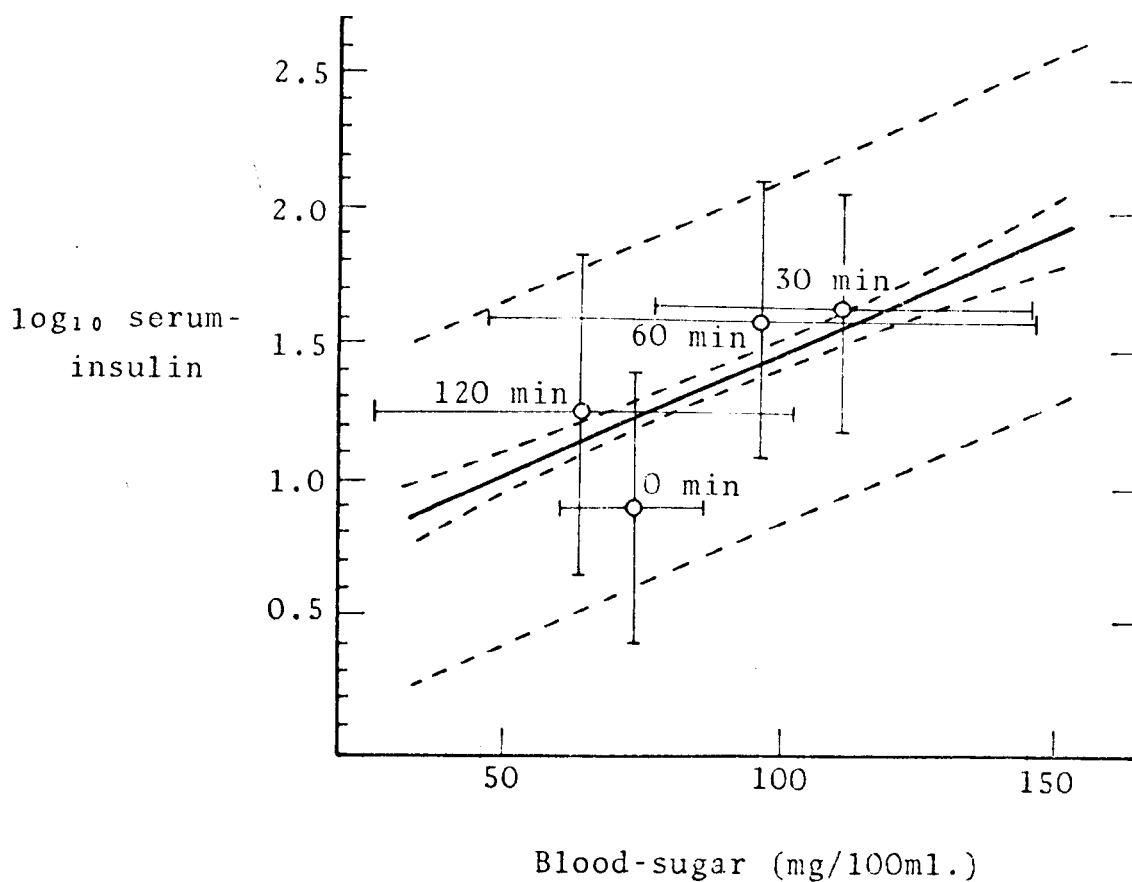


FIGURE 3. Regression of $\log_{10}$ serum-insulin on blood-sugar for 180 paired values of 45 subjects at 0, 30, 60 and 120 minutes of GTT. (see text). For each sampling time the mean points of the group ± 2 SDs are shown. The 95% confidence limits of the regression line and of points in the regression are indicated by dotted lines.

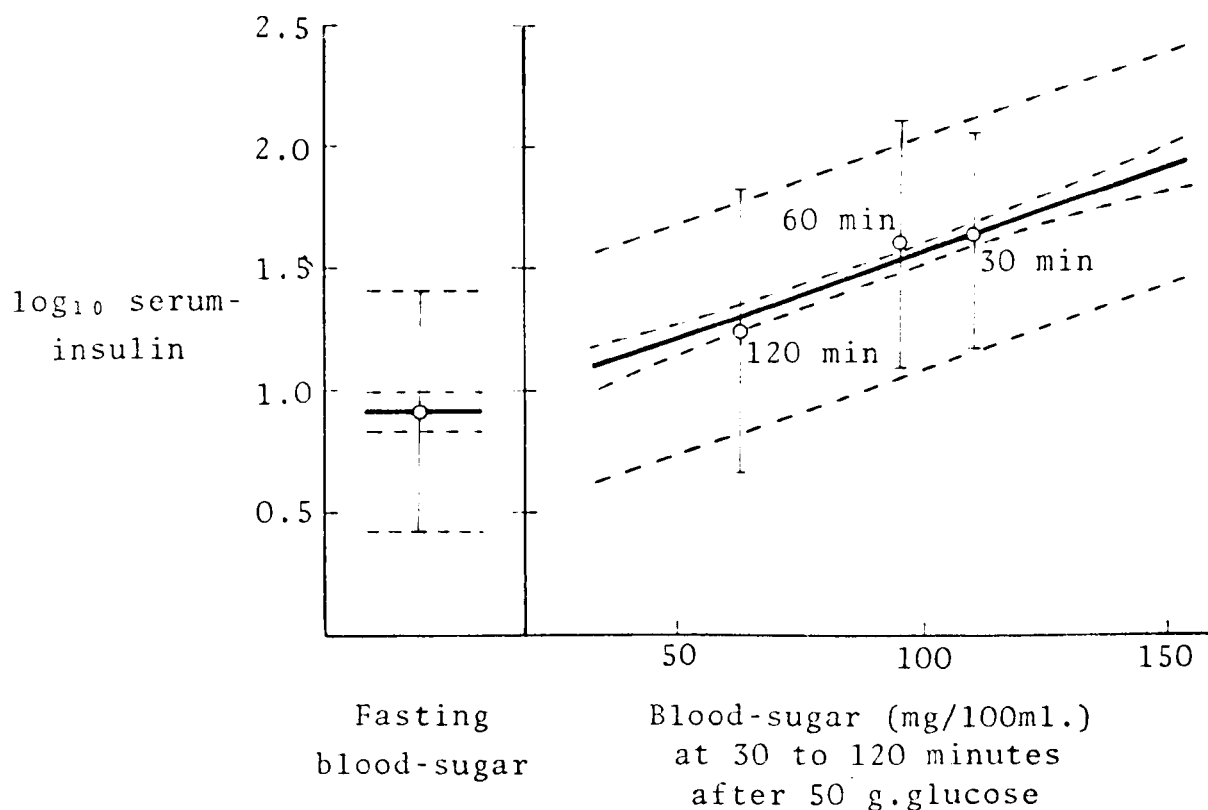


FIGURE 4.

- (A) Mean $\pm$ 2 SD range of fasting $\log_{10}$ serum-insulin in 45 healthy subjects.
- (B) Regression of $\log_{10}$ serum-insulin on blood-sugar for 135 paired values of 45 subjects at 30, 60, and 120 minutes of GTT. The mean $\log_{10}$ serum-insulin $\pm$ 2 SDs at each sampling time is shown for the corresponding mean blood-sugar level. The 95% confidence limits of the regression line and of points in the regression are indicated by dotted lines.

CHAPTER 4

SERUM INSULIN IN PATIENTS WITH VASCULAR DISEASE

INTRODUCTION

In the course of clinical study of patients found to have diabetes while on benzothiadiazine diuretic therapy, it was observed that non-diabetic hypertensive subjects tended to have excessive serum-insulin levels, with the delayed peak response characteristic of the "high insulin secretors" of the normal group. Because of the possibility that this represented an "insulin resistance" similar to that found in simple obesity, the study of "thiazide diabetes" was extended to include patients with normal glucose tolerance and established vascular disease. Of the patients available in hospital practice, those under investigation for hypertension and for intermittent claudication were suitable, since they were ordinarily ambulant and their health had not been otherwise impaired by recent acute episodes such as myocardial infarction. This study therefore reports the results of tests on patients with final diagnoses of essential hypertension or peripheral vascular disease and with normal glucose tolerance; together with tests on a group of essential hypertensives having had long term diuretic therapy who were found to be diabetic.

Interest in the possible biochemical basis for vascular diseases had been dominated by the association between elevated serum cholesterol and coronary heart disease, first demonstrated in a population study in Framingham U.S.A. (Dawber et alii 1957). Subsequently, the observation that patients with past myocardial infarction had an increased frequency of impaired glucose

tolerance, either on intravenous testing (Waddell and Field (1960) or following an oral glucose load (Sowton 1962) suggested that derangements of carbohydrate as well as lipid metabolism could be discerned. The excessive occurrence of the synalbumin insulin antagonist in non-diabetic patients with myocardial infarction (Vallance-Owen and Ashton 1963) supported this concept. Finally, convincing evidence came from epidemiological studies that hyperglycaemia was significantly associated with hypertension, coronary disease and claudication (Ostrander et alii, 1965; Keen et alii, 1965).

Thus it seemed important to determine in a suitably large group of vascular disease patients whether consistent changes in serum-insulin could be observed to account for the increased tendency for hyperglycaemia, and whether these were of sufficient magnitude to represent a possible "risk-factor" for further study. In addition, the mechanism of diabetes occurring in treated hypertensive subjects was investigated.

METHODS

TEST PRODECURE:

The standard 50g. glucose tolerance test was performed on all subjects, as for the normal volunteers, seated in a quiet room or in bed in the ward, after an overnight fast. The majority of hypertensive patients were instructed to take a 300g. carbohydrate diet for 2 - 3 days before testing; and although no such precaution was observed for the peripheral vascular disease subjects, a diet analysis of 5 of the 7 selected patients indicated probably adequate intakes (150 to 364gm. carbohydrate daily, mean 230gm.). Venous blood samples were obtained fasting and at 30, 60

and 120 minutes after the glucose load for blood-sugar and serum-insulin estimation. The insulin levels for vascular disease subjects were measured in assays 1 to 5, 9, and 11. The mean value obtained for the standard serum ("S") was 54 μ U/ml., standard deviation 3.9, which corresponds closely to the quality control data from the assays for the healthy volunteers.

PATIENTS.

(1) Essential hypertension with normal glucose tolerance tests (GTTs).

Inpatients or outpatients who were attending the Hypertension Clinic for investigation and treatment were tested. All had a persistent elevation of diastolic blood-pressure above 110 mm. Hg. Of 27 such subjects, 19 were chosen for study, on the basis of normal GTT (fasting venous blood-sugar less than 100mg/100ml; and after 50g glucose, 120 minute blood-sugar less than 120mg/100ml) and no primary cause for the hypertension. 4 patients were excluded because of abnormal GTTs and 4 because primary renal or adrenal lesions were present (2 patients had pyelonephritis, 1 had gout with renal failure, and 1 had Conn's syndrome). Duration of known hypertension in the selected group ranged from 6 months to 14 years (mean 4 years). 9 of the patients were receiving no therapy, 5 others having had benzothiadiazine diuretic drugs and 5 had other hypotensive drug treatment. 6 patients had renal disease that was not considered to be the primary cause of the hypertension - 1 patient had asymptomatic bacteriuria, 1 had non-significant renal-artery stenosis, 1 had a small kidney on intravenous pyelography; and 3 patients had elevated blood-ureas of 47, 52, and 125 mg. per 100 ml. Clinical details are given in Table 1 which also shows the sex, age, percentage ideal weight and

severity of hypertensive retinopathy (Keith grades 0 - 4) of these subjects.

(2) Peripheral vascular disease.

8 patients with intermittent claudication and subsequently proven vascular occlusion who were admitted for angiography and other investigations by the Peripheral Vascular Disease Clinic were studied. 1 patient was excluded because of overt diabetes mellitus. The remainder were non-diabetic by the above criteria.

(3) Essential hypertensive subjects on long-term diuretic therapy with impaired glucose tolerance.

The plan of this separate collaborative study has been described elsewhere (Dollery et alii 1965, Breckenridge et alii 1967). The aims were to assess the frequency of diabetes in patients receiving long-term benzothiadiazine therapy, and also to obtain more detailed information on the mechanism of their diabetes using the immunoassay of insulin. Of 40 patients attending the Hypertension Clinic, all of whom had been treated continuously with standard doses of benzothiadiazine diuretics for at least 3 years, 12 were found to have an abnormal GTT on the basis of capillary blood-sugar levels exceeding 100mg/100ml fasting or 120mg/100ml at 2 hours after 50g glucose. Of these 12 subjects, 10 agreed to, and were available for, further GTTs, during which venous samples were taken for serum-insulin and blood-sugar measurement. On the basis of the second GTT result, patients were classified as "borderline" diabetics if their venous blood-sugar level at 2 hours was below 120mg per 100ml; as "mild" diabetics with 2 hour venous blood-sugar levels between 120 and 200mg/100ml; and as "moderate" diabetics if the 2 hour venous blood-sugar level exceeded 200mg/100ml. 7 of these patients were eventually re-tested 3 months after cessation of all forms

of diuretic therapy, although they continued other antihypertensive drugs (guanethidine or methyldopa). The body-weights of the diabetic patients ranged from 78 to 124% of ideal for age and height (Kemsley 1951).

RESULTS

I. NON-DIABETIC SUBJECTS WITH VASCULAR DISEASE.

COMPARABILITY OF GROUPS.

Table 1 lists the GTT data and Table 2 contrasts the mean blood-sugar levels, the ages, and the percentage ideal body-weight of the hypertensive and claudication patients with those of the 45 healthy lean volunteers previously tested. The two vascular disease groups have (a) slightly higher 30 and 60 minute blood-sugar values; (b) considerably higher age ranges; and (c) a few more obese patients - 3 hypertensive patients and 1 peripheral vascular disease patient whose weights (120-137%) were above the maximum 115% ideal body-weight of the healthy controls.

SERUM-INSULIN.

The GTT serum-insulin levels are shown in Figure 1 for the hypertensive and peripheral vascular disease subjects in comparison with the normal range of log serum-insulin values of the healthy volunteers. The hypertensive patients have distinctly higher insulin levels at all sampling times, with a similar tendency evident in the peripheral vascular disease group at 30 and 60 minutes. Table 3 compares the mean levels of log serum-insulin with those of the healthy volunteers and indicates that the elevation of serum insulin is highly significant ($p < 0.001$) in the hypertensive group at all sampling times and significant in the peripheral vascular disease group ($p < 0.01$) at 60 minutes. Furthermore, separate comparisons between the 10 youngest hypertensive

patients and the 14 older healthy controls (these groups having similar age ranges) showed significant differences in three of the four sampling times ($p < 0.05$ to < 0.01). Table 4 also shows the same trend to elevated insulin values in hypertensive patients on no therapy (9 subjects) and those with no evidence of a renal lesion (13 subjects). Thus age, drug treatment, or renal disease could not be identified clearly as single factors causing high insulin levels in hypertensive patients.

EFFECT OF AGE

In the 45 normal subjects, age showed no significant association with serum-insulin at any sampling time, and the regression of age on the $\log_{10}$ "integrated insulin area" (or mean GTT serum insulin) gave a slight negative slope not significantly different from zero. However, when the "integrated insulin area" for all vascular disease subjects is plotted against age (Figure 2), the values for essential hypertension and peripheral vascular disease yield a very suggestive positive correlation with age ($r = 0.47$, $p < 0.02$). Figure 2 also indicates that patients with vascular disease within the age range of the healthy controls tended to have higher mean insulin values.

EFFECT OF BLOOD-SUGAR LEVELS.

Despite similar ranges of fasting blood-sugar levels it is evident (Figure 1) that the hypertensives have elevated fasting insulin values compared with the control group. After glucose, it is possible that the comparative hyperglycaemia of vascular disease determines the higher insulin values. In Figure 3, paired $\log_{10}$ serum-insulin and blood-sugar levels are plotted from vascular disease subjects at 30, 60 and 120 minutes of the GTT in relation to the 95% fiducial limits of the linear regression of these

values for normal subjects. The hyperinsulinism of vascular disease appears to be largely independent of blood-sugar levels (except perhaps at levels exceeding 150mg/100ml.).

The qualitative aspects of the serum-insulin response are assessed in Table 4 with respect to the delayed peak levels observed. Whereas 28 (62%) of the 45 normal subjects exhibited their maximum insulin value at 30 minutes after glucose, only 4 (21%) of the 19 hypertensives ($p < 0.001$) and 2 (29%) of the claudicants ($p = 0.1-0.2$) showed this phenomenon. If the 19 hypertensive subjects are divided into tertiles according to their "integrated insulin area" it is seen that the delayed peak insulin phenomenon (Figure 4) is more exaggerated in the upper tertile group, and that this sub-group had considerably higher mean blood-sugar levels at every sampling time.

II. DIABETIC SUBJECTS WITH VASCULAR DISEASE.

(i.e. essential hypertensives on long term diuretic therapy with abnormal screening GTT).

GLUCOSE TOLERANCE AND SERUM-INSULIN.

12 of the 40 patients were found to have abnormal glucose tolerance in the initial screening test, an occurrence of diabetes, approximately twice that observed in an age/sex stratified random sample of Bedford (Breckenridge et alii 1967). This report concerns the results from repeated glucose tolerance tests on 10 of the 12 patients. The venous blood-sugar and serum-insulin levels are listed in Table 5. The 10 subjects were tested while still taking benzothiadiazine diuretics, and 7 of these were then re-tested 3 months or more after all diuretic drugs had been ceased. The mean $\log_{10}$ serum-insulin levels of sub-groups of the 10 patients, according to their diabetes classification as "borderline" (4 subjects), "mild" (3 subjects) or "moderate" (3 subjects) are shown in Figure 5, in contrast

with the mean values for the 19 normoglycaemic hypertensives and the 45 normal volunteers. The mean fasting insulin value of the 10 diabetic hypertensives (log-mean 17 $\mu\text{U/ml.}$) was higher than that of the 45 healthy controls (log-mean 9 $\mu\text{U/ml.}$) and this was statistically significant ($p < 0.01$). The "borderline" diabetic group showed an exaggerated rise of mean serum-insulin at 30 to 120 minutes after glucose, whereas the "mild" diabetic group had a more sluggish but greater than normal insulin response with a delayed peak level at 60 to 120 minutes. The "moderate" diabetic group showed the highest fasting level of insulin with a negligible response following oral glucose. The total insulin response in the 6 borderline/mild diabetics was significantly greater ($p < 0.01$) than in the healthy controls but no differences of statistical significance could be discerned between the insulin values of the diabetic group or sub-groups compared with the 19 normoglycaemic hypertensive patients.

Following cessation of all diuretic therapy, an improvement in glucose tolerance was obvious in one patient (No.1 in Table 5), whose 2 hour blood-sugar level fell from 250 to 110 mg/100 ml., thus changing from a moderate to a borderline diabetic by our classification. This subject also showed an augmented insulin response whereas previously no change in insulin levels following glucose had been observed. In the 6 remaining patients, the diabetic classification did not improve when re-tested. Their serum-insulin levels in both GTTs also were similar, and comparison of the "integrated insulin area" of these subjects showed no marked alteration (Table 5 and Figure 6) after diuretics had been ceased. For these 6 patients, the mean integrated insulin area ($\pm$ one standard deviation) were - on diuretics $128 \pm 77 \mu\text{U/ml/2 hours}$; and off diuretics $128 \pm 66 \mu\text{U/ml/2 hours}$.

THE INSULIN-LIKE-ACTIVITY of various sera from normal and hypertensive subjects was compared with immunoassay levels

of insulin, in order to determine whether the elevated levels measured represented a true increase in biologically active insulin. The "suppressible" fraction of serum insulin-like-activity (SILA) was calculated, using the method of Samaan et alii (1963)*. The results (Table 6) from sera of 4 of the healthy volunteers, 1 non-diabetic hypertensive and 3 diabetic hypertensives, showed that "suppressible ILA" usually exceeded insulin levels measured by immunoassay and had a wide scatter, but when both parameters are plotted on a logarithmic scale (Figure 7) there appears to be a similar correlation for normal and for hypertensive subjects, and there is no evidence for the insulin of hypertensives (whether diabetic or non-diabetic) lacking biological activity on rat adipose tissue.

*Dr. Iris Trayner kindly performed the bioassays.

DISCUSSION

Normoglycaemic patients with essential hypertension (other causes of hypertension being reasonably excluded) or with radiographically proven peripheral vascular disease, clearly showed a substantial elevation of serum-insulin levels, with a delayed peak response, as compared to the group of healthy volunteers. The patient group differed from the "controls" in other respects, however, by having higher mean blood-sugar levels, a tendency to increased body-weight, and greater mean age. Blood-sugar levels "determine" serum-insulin response in normal subjects but the mild relative hyperglycaemia of the vascular disease patients did not account for the elevated insulin values; nor did the extent of deviation from ideal body-weight in these groups seem of sufficient magnitude to explain this. The rise of serum-insulin with increasing age in the disease groups was a striking finding; however, age alone did not explain their hyperinsulinism as shown by comparison of the younger hypertensives with the approximately age-matched normal subjects. A tendency for serum-insulin to rise with age

could be a phenomenon specific for vascular disease or it may merely represent an acceleration of the normal ageing process. Crockford et alii (1966) reported that serum-insulin levels diminish with age, and that this would account for the impaired glucose tolerance of older subjects; whereas Chlouverakis et alii (1967) demonstrated higher serum-insulin in the older subjects of a population sample selected with normal blood-sugar levels.

The study of hypertensive patients on long-term diuretic therapy was commenced so that an estimate of the prevalence of drug-induced diabetes might be made (Dollery et alii 1965), because of reports that benzothiadiazines could aggravate hyperglycaemia (Wilkins 1959, Finnerty 1959). The mechanism appeared to be by suppressing insulin release (Dollery et alii 1962, Samaan et alii 1963). Glucose tolerance improved after cessation of the drugs (Landgon and Wolff 1962, Samaan et alii 1963). Although potassium deficiency in animals impaired glucose tolerance (Sagild et alii 1961) and potassium repletion in patients on thiazides improved hyperglycaemia (Rapaport and Hurd 1964), no direct role of potassium lack in diabetes associated with benzothiadiazmes has been conclusively established (Fajans et alii 1966). 'In vitro' experiments suggested that chlorothiazide exerted a peripheral antagonism to insulin action (Weller and Borondy 1965) but in larger than pharmacological concentrations. At the time of commencing the study, it was not clearly appreciated that the prevalence of hyperglycaemia was increased in hypertensive subjects, but an epidemiological study (Ostrander et alii 1965) as well as the present investigation (1965) gave a strong indication that overt or latent abnormalities in carbohydrate metabolism are a feature of essential hypertension as well as other vascular diseases. Thus the majority of the hypertensive patients treated with long-term diuretics may be "at risk" of

developing diabetes because of their vascular disease rather than because of the treatment. This suggestion would however require a detailed prospective study for confirmation.

Only one patient was observed to show improved glucose tolerance in the present study after cessation of diuretic drugs, and this was associated with a distinct rise in insulin levels after glucose. It seems reasonable to attribute the more severe diabetes to the drug therapy in this case, although spontaneous fluctuations in carbohydrate tolerance are not uncommon. The remaining subjects showed no appreciable change in their diabetes or in their insulin response to glucose following cessation of the drug, and, indeed, 7 of the 9 showed an excessive insulin response by 60 to 120 minutes after glucose rather than any frank suppression of insulin release. It is therefore tempting to regard this group as representing the "diabetic" end of the spectrum of glucose tolerance in hypertension or vascular disease. A clear trend emerges in relation to the fasting insulin levels and the insulin response to glucose (Figure 5), when normoglycaemic healthy subjects and hypertensives are compared with the "diabetic" hypertensives. Thus the fasting serum-insulin shows a progressive rise from normal subjects through normoglycaemic or mild diabetic patients to the moderate diabetic hypertensives. Also, the insulin response, high in the normoglycaemic hypertensives and further elevated in the borderline diabetic group, then shows a more sluggish and subnormal response in the mild and moderate diabetics. This consistent pattern resembles the spectrum of glucose tolerance in obesity, which shares many metabolic aberrations with vascular disease. In both conditions insulin "resistance" manifest by the coexistence of high levels of circulating insulin with normal or mildly elevated blood-glucose levels, appears to precede a progressive failure of pancreatic insulin release as glucose

tolerance deteriorates. In both obesity and vascular disease, the mechanisms of "insulin resistance" remain unexplained, although similar factors to those causing mild hyperglycaemia in the high insulin secretors of the normal group (Chapter 3) must be invoked. No impaired biological activity of the serum-insulin of vascular disease subjects could be discerned on the rat epididymal fat pad, and thus it seems that vascular or humoral factors operate rather than any defect in the secreted insulin. The cause of the impaired secretory response of the pancreatic beta cells is also unexplained, although it appears to be related to the severity of hyperglycaemia, and may have a common mechanism in both obesity and vascular disease.

Sufficient reports are now available of investigations of patients with vascular disease (Peters and Hales 1965; Nikkila et alii 1965; Tzagournis et alii 1967) to designate elevated serum-insulin a "risk factor" worthy of a more extensive evaluation. Thus, serum-insulin measurement might possibly provide further biochemical discrimination of tendency to atherosclerotic disease, since the reported clinical studies indicate a considerable contrast in insulin levels between disease subjects and controls. In addition, large scale studies may provide sufficient numbers of subjects to differentiate the separate effects of age, hyperglycaemia, obesity and other factors, from those of insulin. Finally, insulin, as a "risk factor", may also be an aetiological agent in the genesis of atheromatous lesions, as indicated by preliminary reports (Stout 1968, Mahler 1968) of the effects of insulin on the metabolism of vascular endothelium.

SUMMARY

Significantly raised levels of serum-insulin were found in 19 patients with essential hypertension and 7 patients with peripheral vascular disease, all of whom had normal glucose tolerance. Small increases in blood-sugar levels and in the degree of obesity did not explain the substantial hyperinsulism. The vascular disease subjects showed a significant increase in serum-insulin levels with age. Differences in age alone did not account for the elevated serum-insulin of vascular disease, and no effect of drug treatment or of renal disease was discerned.

A group of 10 hypertensive patients on long-term benzothiadiazine diuretic therapy and with diabetes showed a spectrum of insulin responses similar to that of obese diabetics. With increasing severity of diabetes, the fasting insulin levels progressively rose, and the insulin response to glucose, which was excessive with borderline hyperglycaemia, became delayed and reduced in the mild and moderate diabetics. 1 of 7 cases studied after cessation of diuretic therapy showed improved insulin secretion and glucose tolerance. The remainder had no change in diabetic status or in serum-insulin levels, and it is suggested that their diabetes represents a progression of the "insulin resistance" of vascular disease. Serum-insulin may be a "risk-factor" and an aetiological agent in vascular disease.

TABLE 1. Clinical data and glucose-tolerance test results, with insulin values, in 19 hypertensive patients and 7 peripheral vascular disease patients with normal glucose tolerance.

PATIENT	(AND AGE SEX) (yrs)	PER- CENT IDEAL WEIGHT %	CLINICAL DETAILS				
			DURA- TION (YRS)	SEVERITY (KEITH RETINO- PATHY GRADE:)	DRUGS	RENAL LESION	
ESSENTIAL HYPERTENSION							
1. Hai	(F)	54	102	1	2	-	-
2. Fla	(F)	52	96	10	1	-	-
3. Gow	(M)	44	104	2	3	-	-
4. Ash	(F)	39	96	3	3	α, th.	-
5. Lat	(F)	31	112	1	1	-	-
6. Woo	(F)	58	96	14	1	-	-
7. Bik	(M)	39	88	6	2	α.	-
8. Bic	(F)	35	130	4	1	prop.	-
9. Tub	(M)	43	99	1	1	res.	-
10. Jon	(F)	39	93	2	1	-	-
11. Nim	(M)	33	120	4	2	**	-
12. Har	(F)	58	100	5	2	α, th.	-
13. Hoo	(M)	60	94	6	3-2	th.	-
14. Mit	(M)	55	105	4	3-2	th.	-
15. Kuz	(M)	55	99	8	3-2	th.	-
16. Bay	(M)	52	137	6	4-2	g.	small kidney
17. Has	(M)	63	104	1	(mild)	-	bacteriuria
18. Phi	(F)	45	76	0.5	(mild)	-	renal a.
19. Lan	(F)	46	101	7	(severe)	α, th.	stenosis
							ischaemic
							kidney: blood
							urea 126
PERIPHERAL VASCULAR DISEASE							
1. Ton	(M)	46	124			Claudication	
2. Hol	(M)	74	109			Claudication	
3. Sal	(M)	56	115			Claudication	
4. Bee	(M)	66	106			Claudication	
5. Ric	(M)	68	111			Claudication	
6. Tho	(M)	43	100			Claudication	
7. Fri	(M)	50	92			Claudication	

α = methyl dopa th = Chlorothiazide
 g = guanethidine res = reserpine
 ** = diazoxide in the prop = propranolol
 past

TABLE 1. (continued)

PATIENT	VENOUS BLOOD SUGAR (mins)				SERUM-INSULIN (mins)				* INSULIN AREA"
	0	30	60	120	0	30	60	120	
	(mg/100 ml.)				(μU/ml)				
ESSENTIAL HYPERTENSION									
1.	85	145	145	70	14	56	66	29	96
2.	65	90	100	65	21	41	90	28	107
3.	85	125	140	65	14	74	110	27	137
4.	90	100	105	80	8	89	94	22	128
5.	85	120	120	85	26	94	38	68	116
6.	80	125	135	85	23	69	78	41	119
7.	75	80	70	80	16	35	49	43	80
8.	60	105	100	70	9	45	49	20	72
9.	100	115	160	90	24	67	96	51	137
10.	68	88	98	80	14	150	27	28	113
11.	95	150	125	45	7	41	53	14	69
12.	65	120	80	70	16	110	60	34	121
13.	80	140	130	35	18	68	115	36	143
14.	90	110	115	85	13	60	62	17	88
15.	75	120	105	85	15	66	49	35	91
16.	75	98	95	65	22	132	142	104	230
17.	100	165	210	105	13	53	196	99	226
18.	100	176	175	110	23	108	124	75	190
19.	70	100	100	55	19	69	80	23	111
PERIPHERAL VASCULAR DISEASE									
1.	70	105	113	60	12	44	76	13	89
2.	75	-	165	95	9	178	225	62	291
3.	90	148	185	45	9	68	243	16	227
4.	75	163	140	65	<6	40	69	27	87
5.	80	130	107	63	7	86	133	12	151
6.	70	165	110	50	6	82	53	11	84
7.	95	155	145	105	11	33	32	28	57

* see page

TABLE 2 Mean GTT blood-sugar levels, age and percentage of mean expected weight of the groups of non-diabetic subjects studied.

	45 healthy volunteers	19 patients with essen- tial hyper- tension	7 patients with PVD *
Venous blood-sugar (mg.per 100 ml.) after GTT:			
0min. 	73	81	79
30min. 	112	120	144
60min. 	97	121	138
120min. 	64	75	69
Mean age (yr.) (and range)	31(18-52)	47(32-63)	51(43-74)
Mean % expected weight (and range)	100(78-115)	103(76-137)	108(92-124)

*PVD = Peripheral Vascular Disease.

TABLE 3. Comparison of insulin values in groups and sub-groups of patients with vascular disease against the healthy controls.

Group	Mean log ₁₀ serum-insulin at Time (min.):			
	0	30	60	120
<u>Hypertensive Patients:</u>				
(a) whole group (19 subjects)	1.19 ^{***}	1.84 ^{***}	1.87 ^{***}	1.55 ^{***}
(b) 10 youngest (age range 32 - 46,) (mean 39.5yr.)	1.16 [*]	1.85 [*]	1.81 [*]	1.51 ^{**}
(c) 9 untreated	1.21 ^{**}	1.84 [*]	1.87 [*]	1.58 ^{**}
(d) 13 with no renal lesion	1.18 ^{**}	1.82 ^{***}	1.82 ^{**}	1.50 ^{**}
<u>Peripheral Vascular Disease Patients:</u>				
(e) Whole group (7 subjects)	0.89	1.81	1.97 ^{**}	1.30
<u>Healthy Volunteers (controls):</u>				
(f) Whole group (45 subjects)	0.94	1.64	1.61	1.16
(g) 14 oldest (age range 31 - 47,) mean 34.4yr.)	0.88	1.64	1.62	1.26

When the values for groups (a), (c), (d), and (e) were compared with control group (f), and values for group (b) compared with control group (g), the differences were significant at: $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

TABLE 4. Distribution of normal volunteers and
vascular disease subjects according to
time of peak insulin response.

	NUMBER OF SUBJECTS			
	GTT Serum-insulin			
<u>Group:</u>	<u>peak</u>	<u>at (mins.):</u>	<u>Totals</u>	χ^2 *
	<u>30</u>	<u>60</u>		
(A) Normals:	28	17	45	...
(B) Hypertensives:	4	15	19	7.5 (p<0.01)
(C) PVD:	2	5	7	1.6 (p<0.2)

* Comparison of groups (B) and (C) with group (A)

TABLE 5. Glucose tolerance tests, with insulin values, in patients with essential hypertension, on long-term diuretic therapy, having had previously "diabetic" glucose tolerance. The values in parentheses refer to levels observed 3 months after ceasing diuretic.

PAT- IENTS	DES- CRIP- TION	VENOUS BLOOD-SUGAR				ON THIAZIDE DIURETICS SERUM-INSULIN AT				GTT Insu- lin "Area"*
		AT TIME (min.):				TIME (min.):				
		0	30	60	120	0	30	60	120	
		(mg/100ml.)				(μU/ml.)				
1	Moder- ate	175 (75)	200 (110)	265 (175)	250 (110)	15 (23)	14 (30)	14 (44)	15 (27)	29 (71)
2	"	135 (165)	200 (200)	235 (255)	225 (275)	24 (30)	42 (48)	56 (57)	47 (79)	92 (114)
3	"	300	300	390	374	38	34	44	42	81
4	Mild	130 (120)	226 (190)	216 (210)	183 (195)	<12.5 (<12.5)	49 (17)	33 (22)	22 (36)	63 (56)
5	"	95 (95)	137 (195)	170 (220)	144 (130)	15 (12)	19 (35)	49 (57)	50 (90)	78 (92)
6	"	95	140	195	152	28	53	138	165	220
7	Border- line	85	125	120	110	19	53	37	30	74
8	"	75 (90)	127 (112)	142 (110)	108 (77)	23 (26)	90 (135)	110 (115)	72 (84)	169 (202)
9	"	80 (80)	125 (130)	140 (105)	100 (85)	<12.5 (<12.5)	55 86	74 40	32 22	102 (86)
10	"	80 (85)	160 (200)	195 (190)	105 ...	25 (28)	125 (99)	>200 (165)	95 (74)	266 (216)

* For method of calculation of "GTT insulin area" (i.e. integrated insulin response) see page

TABLE 6. Immunoreactive insulin levels (IRI) compared with "suppressible" insulin-like-activity levels (SILA) on rat fat pad, of serum from healthy volunteers, non-diabetic and diabetic hypertensive subjects (see also Figure

	SERUM-INSULIN (μ U/ml.) at (min.):					
	0		60		120	
	IRI	SILA	IRI	SILA	IRI	SILA
<u>NORMAL VOLUNTEERS:</u>						
Wel	14	38	12	63	10	20 [*]
Koh	14	24	42	40	22	16
Orp	20	28	60	106	17	20 [*]
Ag	14	54	151	1000 [*]	26	28
<u>NON-DIABETIC HYPERTENSIVE:</u>						
Mit	12	27	62	600	17	44
<u>DIABETIC HYPERTENSIVE:</u>						
Jan	15	24	14	48	15	22
Fro	15	20 [*]	49	210	50	560
Web	No test		56	1000 [*]	47	660

* SILA value read at the upper or lower limit of the dose-response line.

● 19 Hypertensive Subjects

○ 7 Peripheral Vasc. Disease Subjects

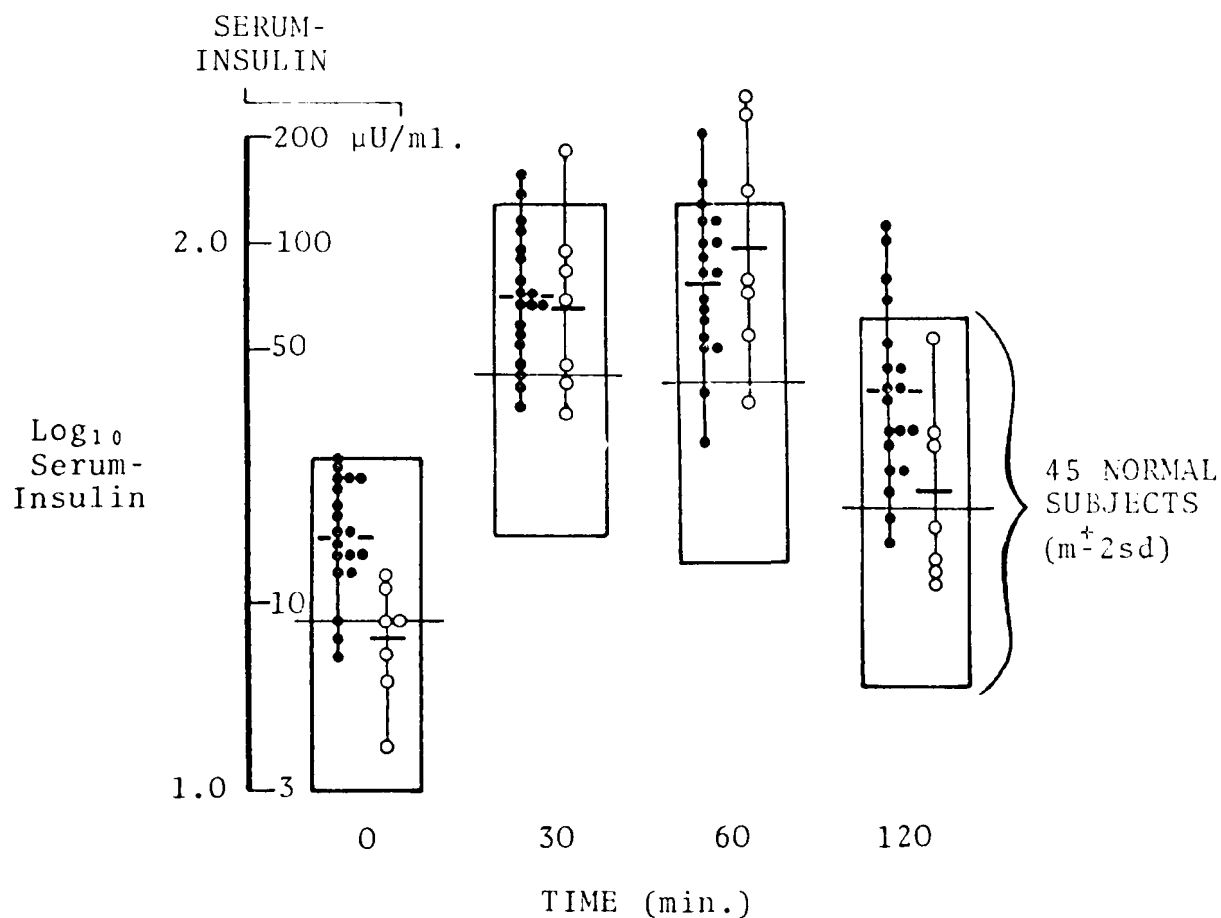


FIGURE 1.

Serum-insulin levels in 19 patients with essential hypertension and 7 with peripheral vascular disease, all with normal glucose tolerance, compared with the normal range from 45 healthy volunteers.

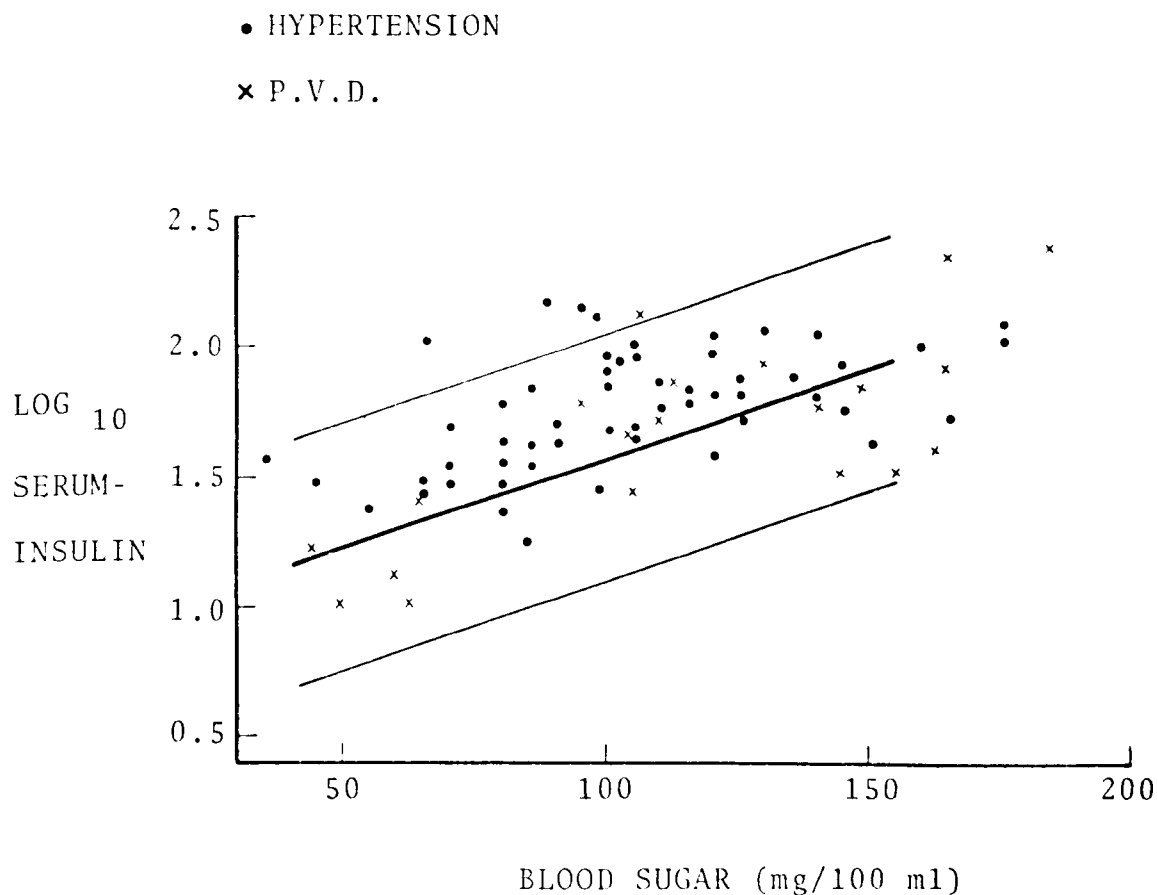
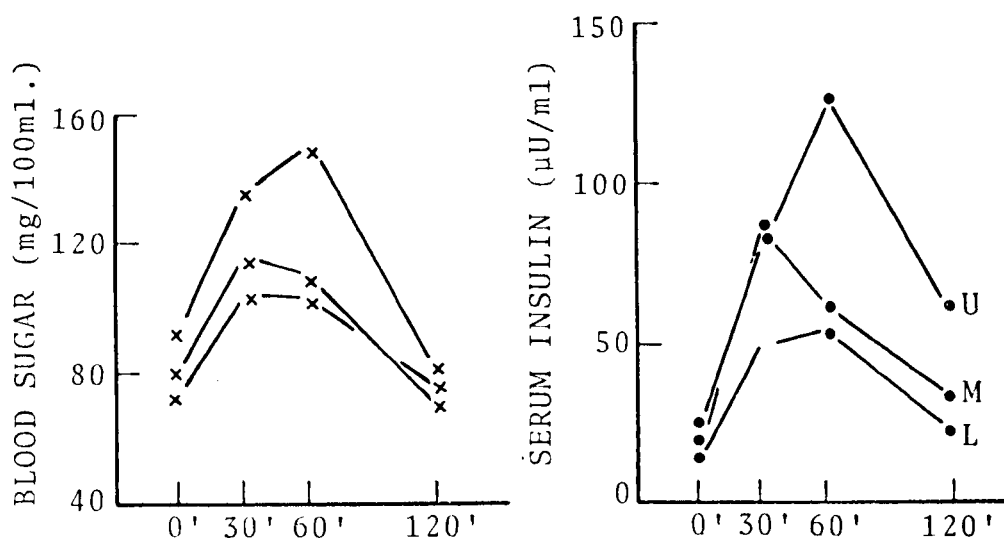


FIGURE 3.

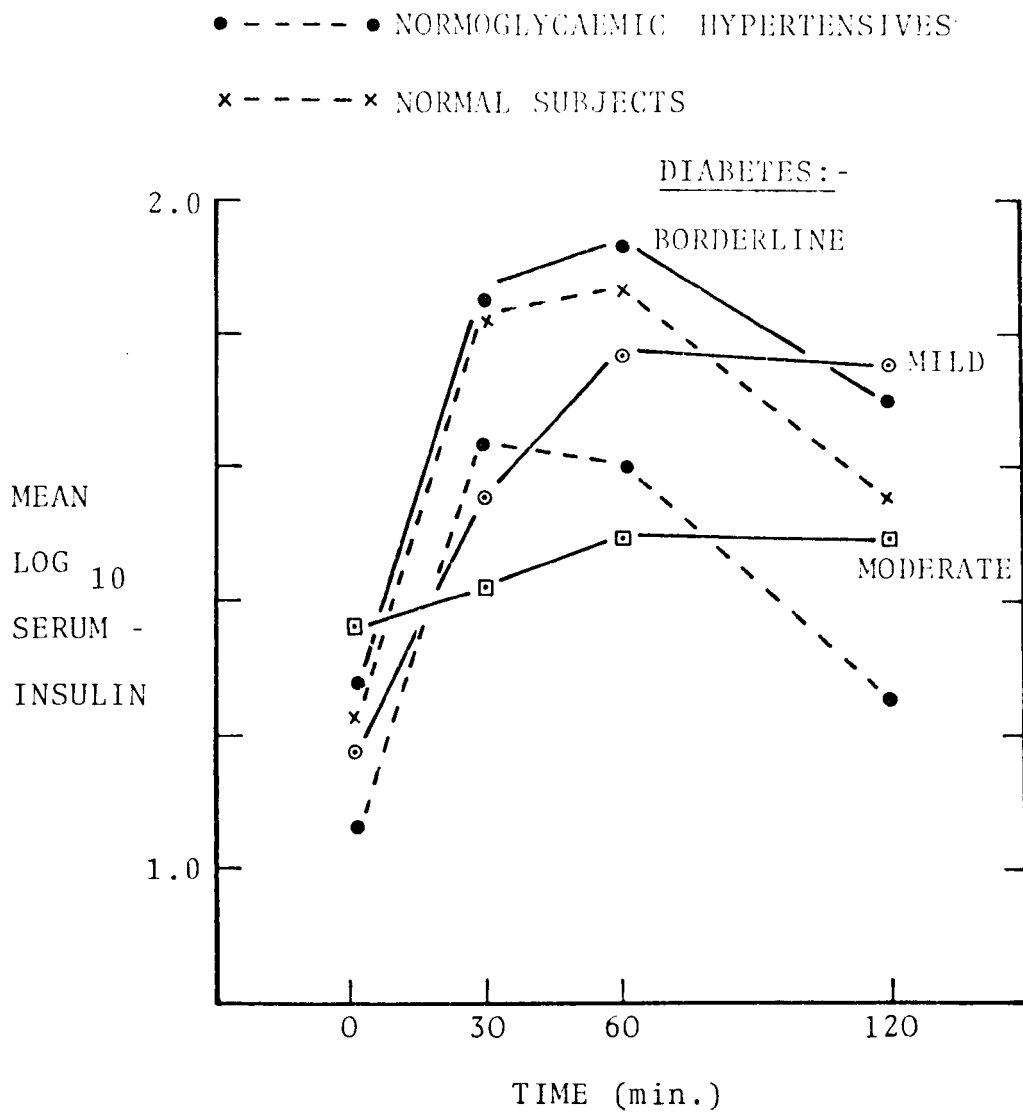
Serum-insulin ($\log_{10}$) and blood-sugar values at 30, 60, and 120 minutes after glucose in hypertensive and peripheral vascular disease subjects, plotted against the 95% fiducial limits for these variables in 45 healthy volunteers. The elevated insulin-levels in vascular disease are independent of the blood-sugar levels.



GTT SAMPLING TIME.

FIGURE 4.

Mean blood-sugar and $\log_{10}$ serum-insulin values for 3 groups of normoglycaemic essential hypertensives, ranked from upper (U), middle (M) and lower (L) tertiles according to the "integrated insulin area". The higher insulin secretors have higher blood-sugar levels and a delayed peak response.



HYPERTENSIVES (ON DIURETICS)

WITH DIABETES OF SEVERITY:-

FIGURE 5. Mean $\log_{10}$ serum-insulin levels for 4 borderline 3 mild and 3 moderate diabetics during glucose tolerance compared to the values for 45 healthy volunteers and 19 normoglycaemic hypertensives.

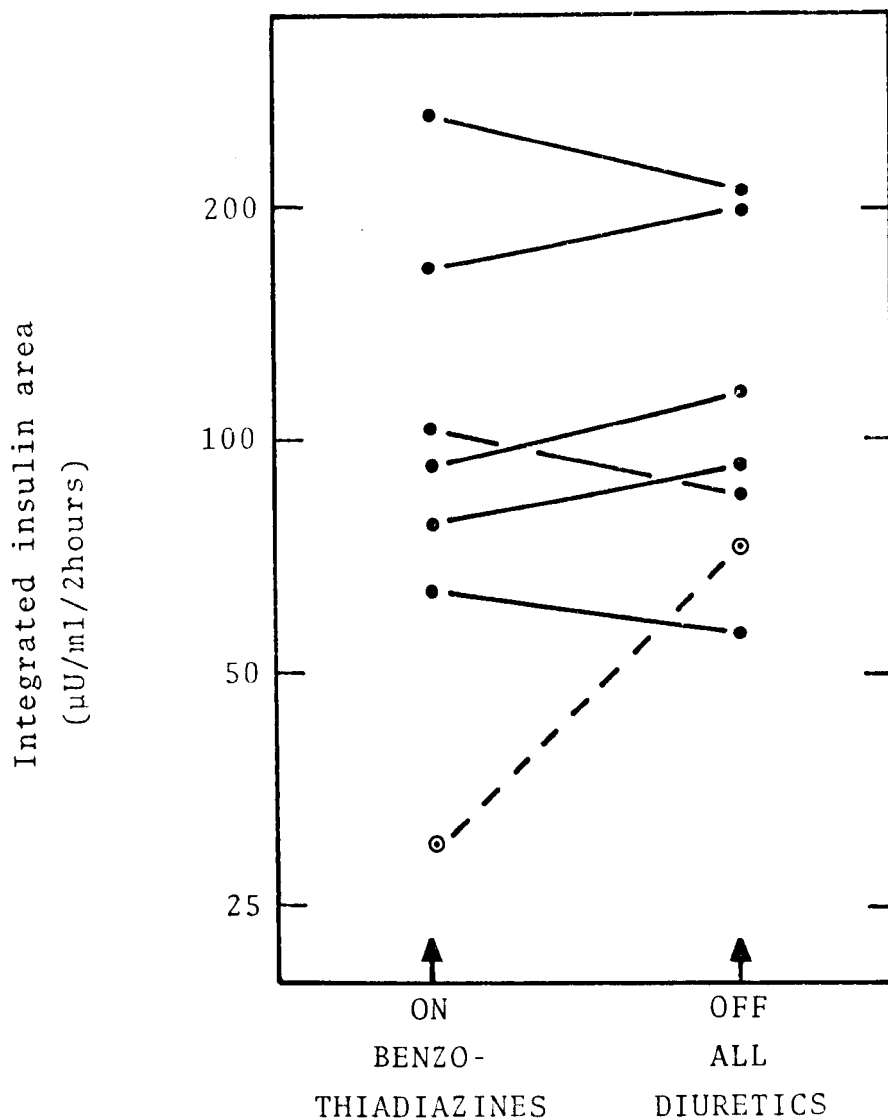


FIGURE 6.

Serum-insulin, expressed as the "integrated insulin area", in 7 essential hypertensives with diabetes while on benzothiadiazines, and 3 months after ceasing all diuretics. One patient ("J") showed substantial improvement in insulin response.

- Normal subjects
- Hypertensive diabetics on thiazides
- × Normoglycaemic hypertensive on thiazides

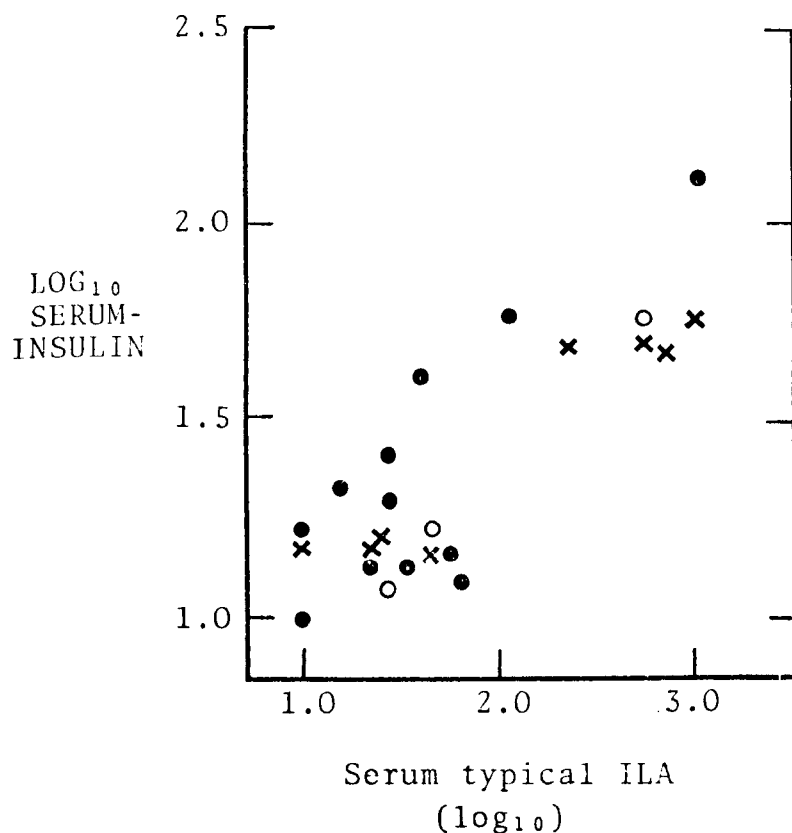


FIGURE 7. Comparison of $\log_{10}$ serum-insulin levels as measured by immunoassay and bioassay, in normal subjects, normoglycaemic hypertensives on thiazides, and diabetic hypertensives on thiazides. The bioassay measurement is of "typical" insulin-like activity on the rat fat pad.

CHAPTER 5

SERUM-INSULIN IN OBESE PATIENTS WITH AND WITHOUT DIABETES, AND THE EFFECT OF STARVATION.

INTRODUCTION

Obesity is generally accepted to be the consequence of overnutrition in normal individuals, but the possibility that it reflects a specific constitutional or metabolic abnormality cannot be refuted. Such a constitution, in fact, has been postulated to have been a survival mechanism in primitive man (Neel 1962), in terms of his ability, preferentially, to conserve carbohydrate and to burn fat stores, particularly during starvation. This same genetic trait in civilised man today is thought to predispose towards the development of diabetes mellitus and atherosclerosis (Albrink 1965): and it is well known that obesity is a common antecedent of diabetes and of cardiovascular disease (Joslin, Dublin, and Marks 1936; Paul et alii 1963).

Increased insulin production in obesity had long been inferred from the observation that the Islands of Langerhans of obese subjects were hypertrophied (Ogilvie 1933) and this was confirmed by the demonstration of elevated levels of circulating insulin in the obese (Karam, Grodsky and Forsham 1963). Resistance to insulin's normal hypoglycaemic action had also been shown by insulin tolerance tests (Arendt and Patee 1956; Fraser et alii 1962) and from forearm studies (Rabinowitz and Zierler 1962) in which the metabolic response of the obese subjects' tissues to intra-arterial insulin administration was blunted. Diabetes occurring in the obese

would appear to represent either an increase in the factors causing "insulin resistance", or alternatively a failure of the pancreatic beta cells to maintain compensatory hyperinsulinism, or a combination of these factors. Karam, Grodsky, and Forsham (1965) favoured the former hypothesis and emphasized the importance of possible insulin antagonists in obese diabetics. Their study showed exaggerated serum-insulin levels in maturity-onset diabetics that correlated roughly with the degree of obesity, the lean diabetics having a clearly deficient insulin response. However, the more severe impairment of glucose tolerance in their lean group was not taken into account. In contrast, Yalow et alii (1965) demonstrated that "mild" and "moderate" diabetes were associated with larger increments of plasma-insulin above the normal than moderate or gross obesity, and suggested that the plasma hyperinsulinism of adult diabetics was not closely related to the degree of obesity.

At this stage, it seemed important to determine in normoglycaemic obese subjects whether a relationship could be discerned between the degree of obesity and the elevation of serum-insulin, so that a basis for identifying any relative insulin deficiency in obese diabetics might be obtained.

The "glucose-fatty acid cycle" as proposed by Randle et alii (1963) provided a unitary hypothesis for the insulin resistance observed in diabetes and obesity. It was suggested that the increased levels of fatty acids and ketone bodies antagonized insulin action on muscle. This theory was supported by detailed "in vitro" studies of animal tissues, and by the observation that severe carbohydrate restriction of healthy subjects resulted in elevated levels of free fatty acids, impaired glucose tolerance, and elevated serum-insulin, all of which mimicked the changes observed in adult diabetics. "Starvation diabetics" had been described by Claude Bernard (1859), and Himsworth (1935) showed that carbohydrate

restriction alone resulted in glucose intolerance and insulin insensitivity. Nevertheless, with the more recent development of insulin assays, conflicting data emerged with respect to the mechanism of starvation diabetes. Thus obese subjects when fasted for 7 days were reported to show a marked rise in serum-insulin-like-activity as measured on the rat fat pad (Samaan et alii 1965) and urine insulin-like-activity also rose (Bolinger et alii 1965). Other workers, however, using the immunoassay, found no appreciable change in insulin levels of the obese after starvation (Yalow et alii 1965; Beck et alii 1964). Finally a diminished insulin secretion had long been suggested by lowering of pancreatic insulin content during carbohydrate lack (Best, Haist, Ridout, 1939). The accepted concept that starvation promoted "insulin resistance" provided a further paradox in that diabetic subjects when fasted generally developed more normal glucose tolerance.

Thus, this study of obese subjects included observations on the effects of a standard period of therapeutic starvation, with the aims of discerning the mechanism of glucose intolerance in fasted normoglycaemic obese subjects, in comparison with the improvement known to occur in fasted diabetic subjects. Glucose tolerance tests, with measurement of endogenous insulin, and insulin tolerance tests that assessed the sensitivity to exogenous insulin, were therefore performed on obese subjects before and after a 7-day period of complete calorie restriction.

METHODS

PATIENTS

21 subjects attending the Outpatient Clinic and requiring treatment for obesity were admitted to hospital for investigation and for therapeutic starvation. 14 patients (13 females and 1

male) without known diabetes were studied, and normal glucose tolerance tests (GTT's) were demonstrated during the investigation. One female ("Bri") was studied on two occasions about six months apart, so that a total of 15 studies on non-diabetic obese subjects were completed. The mean age of the non-diabetic obese subjects was 29 years (range 18 - 55 years) and the mean percentage expected weight was 169% (range 125 - 238%) as calculated from the tables of Kemsley (1951). 7 patients (6 females and 1 male) with obesity were previously known to be diabetic, and this was confirmed by GTT in all but one subject ("Jon") who had been on a diet and showed elevated fasting, 30 and 60 minute blood-sugar levels but a normal 120 minute value. None had received insulin therapy. The mean age of the diabetic patients was 54 years (range 44 - 67 years) and the mean percentage expected weight (Kemsley 1951) was 152% (range 124 - 176%).

CLINICAL STUDY.

All patients received a routine medical examination on admission and freedom from other obvious disease was checked. The general plan of the study was as follows: -

Days 1-3 : 300 grams carbohydrate diet daily
Day 4 : Augmented insulin tolerance test, followed by normal diet.
Day 5 : Oral glucose tolerance test (50 grams), followed by total starvation.
Day 12 : Oral glucose tolerance test (50 grams).
Day 13 : Augmented insulin tolerance test, followed by normal diet.

The augmented insulin tolerance test (Fraser et alii 1962) was chosen because the obese subjects were anticipated to show at least moderate insulin resistance, particularly following starvation. An intravenous insulin dose of 3.3 units

per kilogram by weight or 11.1 units per square metre of body surface area was administered before starvation and the same dose repeated after starvation. Venous rather than the recommended capillary blood samples were obtained. During the period of starvation the patients remained ambulant and were allowed only non-calorie fluids. Urine ketones were checked by nursing staff daily. One patient ("Lam") whose urinary ketone titre diminished suspiciously in the middle of the starvation period had consumed a block of chocolate, but in all other instances the fast was believed to be complete. Venous samples were measured for blood sugar content on the Autoanalyzer (Hoffman 1937) and the serum deep-frozen for insulin assay. Where sufficient volumes remained of the fasting sera, the level of free fatty acids were estimated by Duncombe's method (1964) and serum growth hormone by immunoassay (Hartog et alii, 1963), in the laboratory of Dr A.D.Wright.

Insulin levels of the obese subjects' sera were measured in assays 10 and 12, which gave somewhat higher values for the standard serum used for quality control (see page 69). In order to minimize the possible effect of between assay variability when comparing insulin levels of the obese with the normal subjects, a correction factor of ($\times 0.89$) has been applied to the former's absolute insulin values, as indicated in the text. All other results quoted use the actual assay levels without correction. Each subject's samples for before and after starvation were measured in adjacent tubes of the same assay.

RESULTS

Clinical details and glucose tolerance test (GTT) results for the 15 non-diabetic obese studies and for the 7 diabetic obese subjects are shown in Table 1 and summarized

in Figure 1. Blood samples were obtained from all subjects at 0, 60, and 120 minutes of the GTT and these values only are used in subsequent statistical analyses and correlations.

GLUCOSE TOLERANCE AND SERUM-INSULIN.

A comparison of GTT blood-sugar and $\log_{10}$ serum-insulin values of the obese patients with the 45 leans healthy volunteers is shown in Table 2. Despite their considerable excessive weight, the blood-sugar levels of the non-diabetic obese were similar to the controls, except that the mean 120 minute level was elevated ($p < 0.05$) but well within the normal range. Their $\log_{10}$ serum-insulin values were elevated and significantly different from the controls at all sampling times (even though "corrected"). When the actual $\log_{10}$ serum-insulin values with corresponding blood-sugar levels at 0, 60, and 120 minutes are submitted to linear regression analysis, a highly significant correlation emerges, and the fiducial limits have been calculated for subsequent comparisons. The equation for the 45 paired samples is :

$$(\log_{10} \text{ serum-insulin}) = 0.97 + 0.01x(\text{blood-sugar (mg/100ml)}) .$$

correlation coefficient (r) 0.64, $p < 0.001$

The 7 obese known diabetics, with one exception, showed frankly abnormal GTTs. The fasting $\log_{10}$ serum-insulin was significantly greater than the controls ($p < 0.001$) and the 60 minute value less than the non-diabetic obese ($p < 0.05$) despite the marked hyperglycaemia and comparable degree of obesity (Table 2). When their insulin values are plotted against the blood-sugar levels (Figure 2) no relationship emerges, but it is seen that in comparison with the 95% confidence limits of points in the regression of these variables for the non-diabetic obese, the diabetic group tend to show relative insulin deficiency.

DEGREE OF OVERWEIGHT AND AGE.

Within the group of 15 non-diabetic obese, no significant relationship with $\log_{10}$ serum-insulin and percentage expected weight emerged, (correlation coefficients at 0, 60, and 120 minutes being -0.03, +0.03, and -0.21 respectively) unless the "corrected" data was combined with that of the 45 lean normal subjects (correlation coefficients for 60 pairs of observations at 0, 60, and 120 minutes being +0.67 ($p < 0.001$), +0.56 ($p < 0.001$), and +0.45 ($p < 0.001$) respectively). The relationship in these data, however, is not linear, as shown, for example with the 60 minute sample values in Figure 3, and no general rules can be derived relating degree of overweight and degree of hyperinsulinism. No significant correlations with age and insulin values were obtained in the 15 non-diabetic obese alone or when combined with the 45 lean controls.

THE EFFECT OF STARVATION.

The results of GTTs with insulin levels before and after 7 days' fasting are compared in Table 3 and Figure 1. The non-diabetic obese showed a uniform fall in basal blood-sugar after starvation ($p < 0.001$) but had elevated mean blood-sugar levels at 60 minutes ($p < 0.05$) and 120 minutes ($p < 0.001$). 10 of these 15 patients had 120 minute blood-sugars exceeding 120 mg/100ml and could therefore be classified as "diabetic". Their basal serum-insulin values clearly fell to lower than pre-starvation levels ($p < 0.001$), and at 60 minutes after oral glucose, despite higher mean blood-sugar levels, serum-insulin remained significantly less ($p < 0.01$). A close correlation between $\log_{10}$ serum-insulin and blood-sugar continued; the linear regression equation for 45 paired samples at 0, 60, and 120 minutes being:

$$(\log_{10} \text{ Serum-Insulin}) = 0.94 + 0.0071x \text{ (blood-sugar (mg/100ml))}$$

Correlation coefficient (r) 0.84, $p < 0.001$.

The regression lines of $\log_{10}$ serum-insulin on blood-sugar before and after starvation, in the 15 non-diabetic obese, are compared in Figure 4. The 95% confidence limits of each line are shown, and it is clear that $\log_{10}$ serum-insulin, for a given blood-sugar level, tended to be significantly less after starvation. There appears thus to be a diminished responsiveness of the beta cell to circulating glucose.

In the 7 known diabetic obese, blood-sugar levels after starvation showed considerable improvement, but a substantial impairment of glucose tolerance remained as indicated by the mean 120 minute value of 181 mg/100ml. Although serum-insulin levels fell in this group also, the difference is not significant compared to pre-starvation (Table 3). When the relationship between $\log_{10}$ serum-insulin and blood-sugar is plotted, an interesting phenomenon emerges (Figure 5). In association with the lower blood-sugar levels, many more points fall within the 95% range of the non-diabetic patients (post-starvation) and a more consistent insulin response following oral glucose is evident, in contrast with the pre-starvation comparison (Figure 2). In terms of the blood-serum levels, the insulin responses appear to be more "normal", although the patients remain frankly diabetic.

In these diabetic subjects, it was evident (Figures 2 and 5) that frank insulin deficiency existed, as a general rule, above a blood-sugar level of 180 to 200 mg/100ml. This trend also applied after starvation, as shown in Figure 6, which also indicates that within the range of values encountered, the degree of insulin lack was proportional to the height of the blood-sugar levels.

SERUM FREE FATTY ACID AND GROWTH HORMONE LEVELS.

The mean basal FFA levels (Table 4) measured in 13 of

the non-diabetic obese rise considerably after starvation (from 315 to 664 milli-equivalents/litre) as in 4 diabetic obese (from 338 to 635 milli-equivalents/litre). In contrast, no consistent change in basal growth hormone levels was observed in either group, remaining generally in the low range of less than 2 $\mu\text{g/ml}$. No relationship between the FFA levels or the FFA increment and the GTT or insulin tolerance test parameters could be discerned.

AUGMENTED INSULIN TOLERANCE TESTS.

Although some small variation in estimating the insulin dosage for various individuals occurred (either 0.3 units per kilogram or 11.1 units per square metre body surface area) all subjects were given equivalent doses before and after starvation, and for any individual the sampling times were identical. The blood-sugar levels (Table 5) tend to be lower than for the small number of obese subjects previously reported (Fraser et alii, 1962), one factor being that venous rather than capillary blood samples were obtained. Results are summarized in Figure 7. In the non-diabetic group after starvation, the mean basal blood-sugar levels had fallen from 78 mg/100 ml to 65 mg/100 ml ($p < 0.05$) but the remaining observations at 20 to 120 minutes were not significantly different ($p = 0.1 - 0.5$) although after starvation a small tendency for resistance to insulin action may be recognised in that despite significantly lower initial blood sugar levels, the comparable samples at 20 and 30 minutes had slightly higher mean levels. At 60, 90 and 120 minutes, the lower mean blood-sugar levels after starvation suggest a small impairment of the glycogenolytic and gluconeogenetic responses to hypoglycaemia.

The grossly abnormal insulin tolerance of the diabetic obese before starvation related largely to their considerably elevated basal blood-sugars. 7 days fasting resulted in a fall

of the basal levels to within the "normal" range, so that comparison with the non-diabetic may be considered more valid. Comparing the two groups after starvation, the mean blood sugar-levels of the diabetics are elevated at 0 and 20 minutes ($0.1 > p > 0.05$; and $p < 0.02$ respectively) but subsequent values are almost identical with the non-diabetics ($p > 0.5$), indicating a similar level of glucose production in response to hypoglycaemia. Thus although a marginal insulin resistance in the diabetic group may be inferred from the somewhat delayed hypoglycaemic action of administered insulin at 20 minutes, this test uncovers no substantial antagonism to insulin action in either group despite the elevated FFA levels and the ketonuria observed.

DISCUSSION

The association between obesity and elevated levels of circulating insulin has been widely reported (Karam et alii 1963, Beck et alii 1964, Yalow et alii 1965, Perley and Kipnis 1966) and the present study also has shown that both the basal and the glucose-provoked serum-insulin levels in the non-diabetic obese are substantially higher than in lean subjects. Although a significant correlation was obtained between percentage expected weight and the serum-insulin values in the combined data of lean volunteers and obese subjects, within each group, no such relationship emerged. The lack of correlation in the obese may in part have been due to the small number of subjects and the nature of selection for the study. No general rules can be applied from these data. Bagdade and colleagues (1967) found a linear correlation between fasting serum-insulin and percentage deviation from ideal body weight (range 90 to 200%) in 37 male volunteers. This is in accord with the general consensus of opinion that obesity determines hyperinsulinism (rather

than the reverse) as indicated by observations that weight reduction reduces serum-insulin levels (Karam et alii 1963, Salans et alii 1968, Jackson et alii 1969) and that the induction of experimental obesity in volunteers promotes many of the metabolic features of spontaneous obesity including elevated insulin levels (Sims and Horton, 1968).

The assumption is made that the levels of insulin measured in venous serum reflect the secretion rate rather than any alterations in utilization or degradation that may occur in obesity. Supporting this, the studies of Stern et alii (1968) showed that a wide range of body weight and of blood glucose values did not affect the linear relationship found between insulin irreversible loss rate and the plasma insulin concentration under steady state conditions. That hypersecretion of insulin occurs is also supported by the finding of hypertrophied Islands of Langerhans in the obese (Ogilvie 1933). The mechanism whereby overproduction of insulin occurs is not known, but may be secondary to the recognized insulin insensitivity of the peripheral tissues, about which some further suggestive evidence is emerging. Thus, although humoral antagonists of insulin action in obesity have been proposed, such as elevated free fatty acid levels (Randle et alii 1963, Pelkonen et alii, 1968) or possibly increased cortisol production (Cohen 1958) more emphasis recently has been placed on anatomical factors. The diminished fixation of radio-insulin by the forearm muscle of obese subjects may be related to impaired filtration of insulin from the vascular compartment (Butterfield 1965). Adipose tissue sensitivity to insulin is dependent on the size of the adipose cells, which are larger and less responsive in vitro from the obese (Salans et alii 1968). However, the peripheral insulin resistance of obese subjects is not clearly reflected in the levels of blood-glucose, which in the present study were very similar to the levels of lean healthy subjects. The role of hepatic glucose production in

obesity is uncertain, for although increase gluconeogenesis has been assumed from indirect experiments (Arky and Freinkel 1966, Kreisberg et alii 1967) this has not been confirmed (Franckson et alii 1966).

In starvation, the available evidence indicates that the hepatic binding of insulin is diminished (Unger et alii 1963) or unchanged (Genuth 1966). Thus, it is reasonable to attribute the fall in basal insulin levels observed in the starved obese subjects to diminished insulin secretion, rather than increased hepatic extraction. Concurrent studies have confirmed that insulin levels as measured by immunoassay conclusively fall during starvation in normal subjects (Cahill et alii 1966) and the obese (Solomon et alii 1968). It is difficult to support the contention of Madison and colleagues (1965) that the reduction of blood-glucose during starvation depends on ketone-induced insulin secretion; or that of Jenkins (1967) that elevated free fatty acid levels stimulate insulin release. Following oral glucose, two thirds of the starved non-diabetic obese subjects showed "diabetic" tolerance curves, with a delayed and subnormal use of serum-insulin. When related to the blood-sugar levels, significantly lower insulin values in these subjects were apparent. Thus "starvation diabetes" in part must relate to insulin deficiency. The delayed absorption of glucose that occurs (MacKay and Bergman 1933) may, however, contribute to the elevated 120 minute levels of blood-sugar. The fall in insulin content of pancreatic beta cells observed by Best et alii (1939) provides further evidence that insulin lack, rather than insulin resistance, contributes to glucose intolerance after carbohydrate deprivation or starvation. Cahill and his co-workers have proposed that the availability of insulin may be the control mechanism relating to the supply of energy substrates during starvation; its lack promoting the liberation of free fatty acids and ketones for the metabolism of most tissues. Of other hormonal factors in starvation, growth hormone shows inconsistent responses in lean subjects

(Cahill et alii 1966) and little rise in the obese (Beck et alii 1964) as confirmed in this study. There are conflicting reports about the role of glucagon secretion (Unger et alii 1963; Vance et alii 1968). Finally, the non-suppressible insulin-like activity (NSILA) of serum (Samaan et alii 1965) and urine (Bolinger et alii 1965) rises substantially during starvation, and this may represent a further endocrine mechanism, as yet unidentified, that must await the more complete elucidation of the nature of "NSILA".

Augmented insulin tolerance tests in the non-diabetic obese yielded no good evidence for substantially increased insulin resistance after starvation, despite the marked rise of free fatty acids and the starvation ketosis. The large dose of intravenous insulin used could not be considered physiological; but the test was designed to evaluate the activity of acromegaly (Fraser et alii 1962) which is also associated with antagonism to insulin action. A preliminary report (Tzagournis and Skillman 1969) confirms that the starved non-diabetic obese show no further impairment of insulin sensitivity after starvation. It is difficult on this evidence to assign a primary role to circulating free fatty acids in mechanisms of insulin resistance and indeed, the significance of the "glucose-fatty-acid cycle" in man must depend on the pursuit of more direct experimental evidence (a topic reviewed by Ruderman et alii 1969) and perhaps on more sophisticated tests of insulin sensitivity. In the meanwhile, this study suggests that in "starvation diabetes", as with diabetes, impaired insulin secretion plays at least as important a role as insulin resistance.

The known diabetic obese subjects showed lower mean insulin levels than their non-diabetic obese counterparts, and definitely low individual levels in relation to their elevated blood-sugars. The degree of impairment of insulin

secretory response was approximately proportional to the severity of the diabetes, and there is a suggestion in the data that above blood-sugar levels of 180-200 mg/100ml. a predictable decline in serum-insulin might emerge if larger numbers were studied. A similar "horse-shoe" relationship of insulin response with glucose response has been reported by Reaven and Miller (1968). One obvious explanation for this manifestation is that the extent of insulin deficiency determines the degree of hyperglycaemia. However, after starvation, the insulin levels in the diabetics, although lower, were more appropriately related to the blood-sugar in most cases, but nevertheless continued to show a trend to decline with blood-sugar levels above the threshold of 180-200 mg/100ml. This phenomenon of insulin deficiency and hyperglycaemia is further pursued in later studies. After starvation, when the basal blood-sugar levels of the diabetic obese were comparable with those of the non-diabetic patients, the response during augmented insulin tolerance tests was very similar in the two groups. Thus no extraordinary "insulin-resistance" existed in the diabetics, supporting the view that the mechanism of diabetes is essentially related to a defect in insulin production relative to the demands imposed by the presence of obesity. The exact cause of insulin resistance in obesity, and the nature of the stimulus for increased insulin secretion, requires elucidation, as does the progressive failure of function of the pancreatic beta cells with increasing hyperglycaemia.

SUMMARY

A group of obese patients with normal glucose tolerance showed substantial elevations of mean serum-insulin levels compared with lean controls. A close correlation with blood-sugar levels was evident but within the group of obese non-

diabetic subjects, no relationship between the degree of obesity or between age and the serum-insulin levels was found. Following 7 days' total starvation, basal insulin levels showed a significant fall, and the insulin response to glucose for a given blood-sugar level was significantly less than before starvation. Two thirds of these subjects were "diabetic" after starvation. Augmented insulin tolerance tests revealed no increase in insulin resistance after starvation, despite the uniform elevation of free fatty acids and the ketonuria observed. The mechanism of "starvation diabetes" appears to relate mainly to the impaired insulin response to glucose.

Obese patients with known diabetes had a similar elevation of mean basal serum-insulin levels to the non-diabetic obese subjects, but at 60 minutes after glucose the mean serum-insulin level was significantly less. This relative insulin lack was more obvious if the serum-insulin levels were related to the blood-sugar levels. Following 7 days' starvation, the glucose tolerance of diabetics improved, and although serum-insulin levels fell, these were more appropriately related to the blood-sugar levels. Both before and after starvation, blood-serum levels exceeding 200mg/100ml. were associated with a progressive decline of serum-insulin. After starvation, augmented insulin tolerance tests revealed no difference in the insulin resistance of diabetic compared with the non-diabetic obese. Although insulin resistance occurs in obesity, diabetes appears to be determined by impaired insulin secretion.

TABLE 1 Clinical data and results of blood-sugar and serum-insulin estimations during GTT on 15 non-diabetic obese and 7 diabetic obese patients. Post-starvation values are shown in parentheses.

SUBJECTS			AGE (yrs)	HEIGHT (ins.)	WEIGHT (lb.)	PERCENTAGE EXPECTED WEIGHT
<u>NON-DIABETIC OBESE :</u>						
1(A)	Bri	...	19	61	218	193
2	Cof	...	21	65	163	128
3	New	...	32	66	166	125
4	Mui	...	21	65	207	163
5	Bal	...	18	65	161	129
6	Ann	...	28	64	196	156
7	Win	...	18	64	287	238
8	Hol	...	32	60	197	172
9	Ros	...	55	58	178	145
10	Gus	...	38	65	213	159
11	Mat (M) *	...	30	67	212	146
12	Wal	...	43	65	243	175
1(B)	Bri	...	19	61	229	202
13	Liv	...	35	66	276	205
14	Lam	...	30	59	220	198
<u>MEANS :</u>			29	...	...	169
<u>DIABETIC OBESE :</u>						
1	Joh	...	58	59	216	176
2	Kin(M)	...	52	68	227	150
3	Agu	...	51	61	227	173
4	Par	...	54	62	196	146
5	New	...	50	64	173	124
6.	Eva	...	67	66	182	129
7.	Tu	...	44	63	220	165
<u>MEANS:</u>			54	...	...	152

TABLE 1 (cntd)

BLOOD-SUGAR (mg/100ml) AT:				SERUM-INSULIN (uU/ml) AT:					
Omins	30mins	60mins	120mins	Omins	30mins	60mins	120mins		
65(56)	-	-	75(145)	65(135)	27(19)	-	-	82(68)	45(60)
60(60)	90(95)	80(135)	70(165)	31(18)	115(36)	82(58)	80(175)		
85(50)	85(95)	100(145)	70(145)	30(17)	62(62)	133(77)	33(70)		
90(55)	105(97)	70(130)	85(153)	26(18)	26(42)	145(56)	42(140)		
65(45)	100(111)	110(116)	90(125)	34(28)	133(90)	150(90)	175(77)		
75(65)	113(85)	98(110)	52(125)	58(25)	300(44)	300(33)	39(54)		
77(57)	-	-	110(164)	98(86)	36(13)	-	-	94(121)	78(39)
85(75)	170(120)	135(125)	80(85)	39(32)	270(125)	180(100)	36(46)		
80(50)	75(125)	60(145)	60(158)	26(13)	-	-	57(66)	24(105)	
90(60)	140(110)	120(160)	65(130)	21(11)	63(28)	80(57)	34(47)		
65(46)	-	-	120(133)	82(133)	28(15)	-	-	76(98)	52(59)
60(45)	105(55)	125(100)	115(125)	43(15)	80(29)	100(140)	92(>200)		
80(55)	-	-	115(95)	85(95)	28(28)	-	-	110(74)	18(54)
75(55)	-	-	120(90)	55(75)	32(23)	-	-	>200(71)	32(50)
63(75)	-	-	110(80)	73(75)	20(20)	-	-	100(28)	33(26)
<u>MEANS:</u>				<u>LOG MEANS:</u>					
74 (57)		103(124)	76(121)	31(19)		115(71)	46(68)		
112(85)	200(155)	194(222)	88(150)	41(22)	175(70)	127(77)	50(40)		
97(58)	- (130)	207(145)	145(132)	42(23)	-	-	120(80)	86(44)	
150(41)	246(-)	277(135)	227(140)	33(17)	-	-	43(18)	47(20)	
240(145)	330(225)	310(275)	260(300)	11(12)	23(18)	19(18)	9(21)		
125(60)	230(-)	275(210)	262(221)	21(6)	-	-	15(36)	29(42)	
130(55)	205(95)	260(100)	280(145)	44(9)	60(23)	56(29)	47(54)		
255(100)	404(173)	306(180)	370(182)	33(49)	-	-	74(64)	123(77)	
<u>MEANS :</u>				<u>LOG MEANS:</u>					
158(78)	269(156)	261(181)	235(181)	30(16)	60(31)	50(39)	44(38)		

TABLE 2.

Comparison of mean values for blood-sugar levels and $\log_{10}$ serum-insulin values at 0, 60 and 120 minutes of GTT, and for age and percentage expected weight, in 15 obese non-diabetic patients, 7 obese diabetic patients and the 45 healthy volunteers. For the purposes of this comparison, the insulin values of the obese subjects have been corrected for between assay variability. (See text):

Mean values:

SUBJECTS AND NUMBER	AGE (Years)	PERCENT EXPECTED WEIGHT %	BLOOD-SUGAR (mg/100ml.) at:			"Corrected" LOG ₁₀ SERUM-INSULIN AT:
			0 min.	60 min.	120 min.	
(A) Obese non-diabetic (15)	29	169	74	103	76	1.33 1.83 1.48
(B) Obese diabetic (7)	54	152	158	261	235	1.31 1.51 1.46
(C) Normal lean volunteers (45)	29	100	73	97	64	0.91 1.61 1.26

SIGNIFICANCE OF DIFFERENCES: "p" by students "t" test*						
Obese non-diabetics (A) and normals (C)	...	N.S.	N.S.	p<0.05	(p<0.01)*	p<0.01 (p<0.01)*
Obese diabetics (B) and normals (C)	...				p<0.001	N.S. N.S.
Obese non-diabetics (A) and obese normals	(B) ...				N.S.	p<0.05 N.S.

* Variance ratio ("F" test) is significant.

TABLE 3

Comparison of mean values ($\pm$ 1 standard deviation) for GTT blood-sugar levels and $\log_{10}$ serum-insulin values in 15 obese non-diabetic and 7 obese diabetic patients, before and after 7 days starvation.

Means $\pm$ 1 standard deviation; and "p" by Students "t" test

SUBJECTS (and no.)	BLOOD-SUGAR (mg/100ml) at:			LOG ₁₀ SERUM-INSULIN at:		
	Omins	60mins	120mins	Omins	60mins	120mins
Obese non-diabetic (15)	Before fast	74 $\pm$ 11	103 $\pm$ 22	76 $\pm$ 17	1.49 $\pm$ 0.12	2.06 $\pm$ 0.19
	After fast	57 $\pm$ 9	124 $\pm$ 26	121 $\pm$ 30	1.27 $\pm$ 0.14	1.85 $\pm$ 0.19
Obese diabetic	Before fast	158 $\pm$ 63	261 $\pm$ 41	235 $\pm$ 93	1.47 $\pm$ 0.22	1.70 $\pm$ 0.37
	After fast	78 $\pm$ 36	181 $\pm$ 60	181 $\pm$ 61	1.20 $\pm$ 0.30	1.59 $\pm$ 0.28
SIGNIFICANCE OF BEFORE/AFTER STARVATION DIFFERENCES IN: -						
(A) obese non-diabetic:			p<0.001	p<0.05	p<0.01	N.S.
(B) obese diabetic:			p<0.01	p<0.01	N.S.	N.S.

TABLE 4

Basal levels of serum growth hormone and serum free fatty acids in non-diabetic and diabetic obese subjects. Post-starvation values are shown in parentheses.

SUBJECTS		SERUM HORMONE (mug/ml.)		SERUM (mug/ml.)
NON- DIABETIC OBESE:				
1 (A)	Bri	-	-	315 (664)
2	Cof	<2	(2.5)	350 (650)
3	New	6.5	(<2)	290 (610)
4	Mu	<2	(<2)	290 (510)
5	Bal	3.8	(8.5)	530 (1020)
6	Ann	<2	(<2)	75 (890)
7	Win	<2	(<2)	440 (600)
8	Hol	<2	(<2)	350 (575)
9	Ros	<2	(<2)	190 (660)
11	Mat	<2	(<3)	510 (810)
12	Wal	<2	(<2)	340 (610)
1 (B)	Bri	7.2	(15)	215 (535)
14	Lam	-	-	250 (670)
		Medians:		Means:
		<2 (<2)		315 (664)
DIABETIC OBESE:				
1	Joh	10	(2.3)	70 (760)
2	Kin	<2	(2.0)	250 (640)
3	Agu	<2	(<2)	460 (700)
7	Tu	3.8	(<2)	470 (440)
		Medians:		Means:
		<2/3.8 (<2/2.0)		338 (635)

TABLE 5

Blood sugar data during augmented insulin tolerance tests in 12 non-diabetic and 5 diabetic subjects, before and after 7 days starvation.

SUBJECTS	% EXP. WT.	BLOOD SUGAR (mg%)								AUGMENTED I.T.T.								INTRA- VENOUS DOSE (UNITS)
		BEFORE FAST								AFTER FAST								
		(MINS.)								(MINS.)								
		0	20	30	40	60	90	120	0	20	30	40	60	90	120			
OBESE- NORMAL G.T.T.																		
1(A)	Bri	193	65	20	-	25	30	35	55	70	40	-	25	25	45	60	22.0	
2	Cof	128	45	13	-	25	25	30	40	60	25	-	15	20	20	25	22.5	
3	New	125	80	30	-	10	25	30	45	55	15	-	18	23	25	35	19.0	
4	Mui	163	80	15	17	22	30	42	60	61	36	18	13	23	26	35	22.0	
5	Bal	129	75	30	15	30	30	50	79	59	25	15	13	25	40	46	20.0	
6	Ann	156	75	45	-	30	25	55	60	80	50	-	45	45	50	70	26.0	
9	Nos	145	110	25	25	-	35	40	50	86	36	32	-	38	49	76		
10	Gas	159	95	-	<25	-	40	50	75	60	-	25	-	25	35	35	22.0	
11	Mat	146	65	60	-	30	32	-	58	55	40	-	26	26	-	38	30.0	
12	Wal	175	80	-	<25	-	25	-	55	70	-	50	-	45	-	50	39.0	
1(B)	Bri	202	70	-	20	-	20	25	40	63	-	18	-	15	23	29	29.0	
13	Lin	205	75	-	16	-	32	40	60	64	-	20	-	18	27	31	34.0	
MEANS		161	78	29	17	25	29	40	56	65	33	25	22	27	34	44	(26)	
Comparison of pre-fast with post-fast values in matched ITT's (P by "t" test)										p<.05	p>.5	p>.1	p>.5	p>.2	p>.2	0.1>p>0.05		
OBESE - DIABETIC G.T.T.																		
3	Agu	173	147	42	40	20	25	45	50	76	32	10	10	14	12	19	31.0	
5	New	124	140	94	-	38	<10	-	81	69	54	-	26	35	-	41	19.0	
7	Tu	165	232	210	180	152	135	120	115	160	50	30	25	35	45	65	22.0	
2	Kin(M)	150	114	68	48	-	47	62	73	80	45	25	-	30	35	55	24.0	
1	Woh	176	105	50	33	-	33	52	80	62	53	45	-	28	28	28	18.0	
MEANS		158	148	93	75	70	49	56	80	77	47	28	20	28	30	42	(25)	
Comparison with non-diabetic obese post-starvation values (P by "t" test)										0.1>p>.05	p<.02	p>0.7	p>0.8	p>0.8	p>0.5	p>0.7		

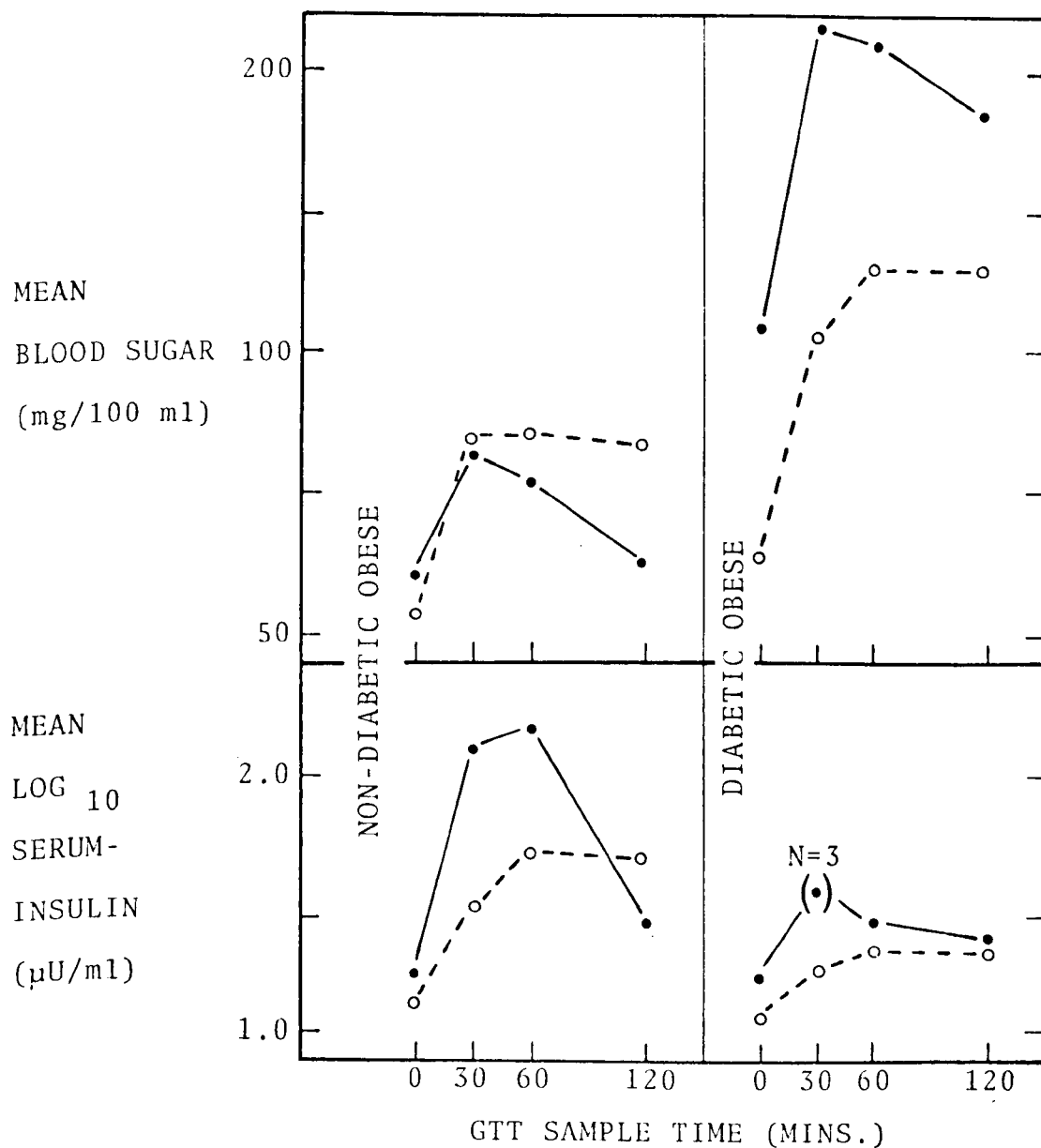


FIGURE 1. Mean values for blood-sugar and $\log_{10}$ serum-insulin during 50g. oral glucose tolerance tests in 15 normoglycaemic obese patients and 7 diabetic obese. Pre-starvation values are shown by the unbroken lines and the values after 7 days total starvation by the interrupted lines.

• = values at 60 and 120 minutes of GTT.

× = fasting values

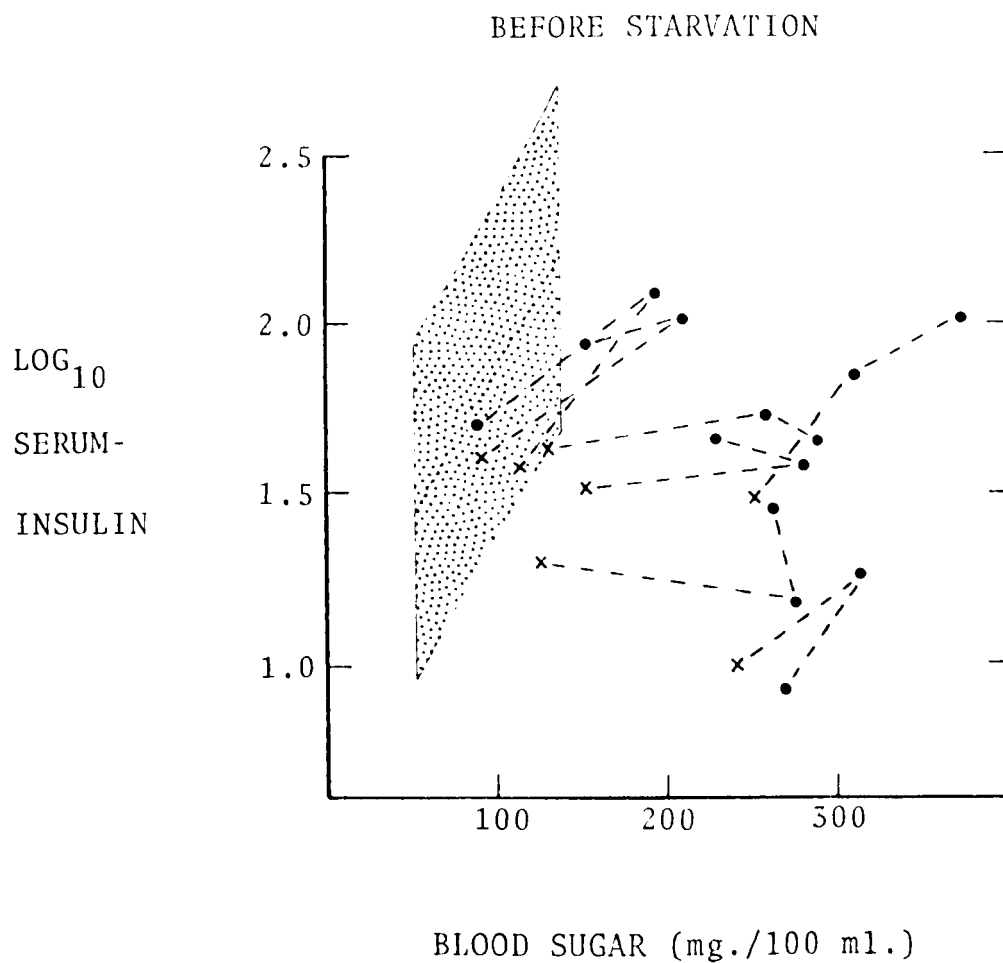


FIGURE 2.

Log₁₀ serum-insulin values of 7 diabetic obese subjects at 0, 60, and 120 minutes of GTT. The group show relative insulin deficiency as compared to the 95% range of values observed in non-diabetic obese subjects (hatched area).

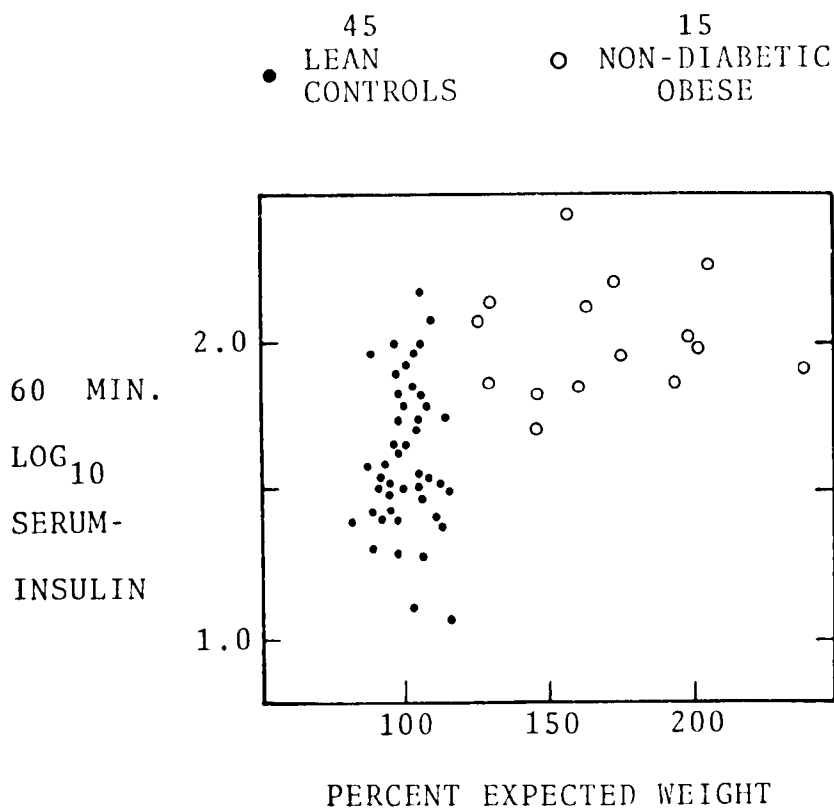


FIGURE 3.

Log₁₀ serum-insulin at 60 minutes of GTT in relationship to percentage expected weight in 45 healthy volunteers (body weight 79-115%) and 15 non-diabetic obese patients (body weight 125-238%). Within groups there is no correlation. (The "corrected" insulin levels for the obese subjects are portrayed.

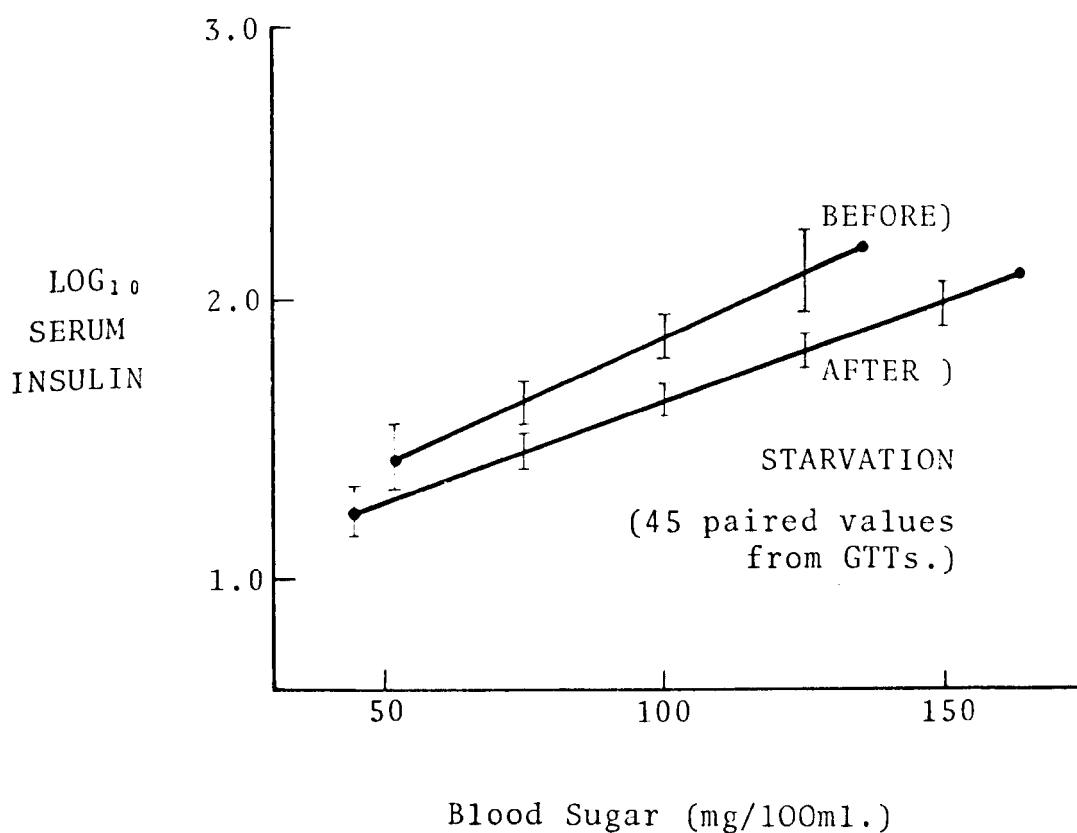


FIGURE 4.

Regression lines of $\log_{10}$ insulin on blood-sugar levels in non-diabetic obese subjects before and after starvation. The bars indicate the 95% confidence limits for each line.

× = fasting values

• = values at 60 and 120 minutes of GTT

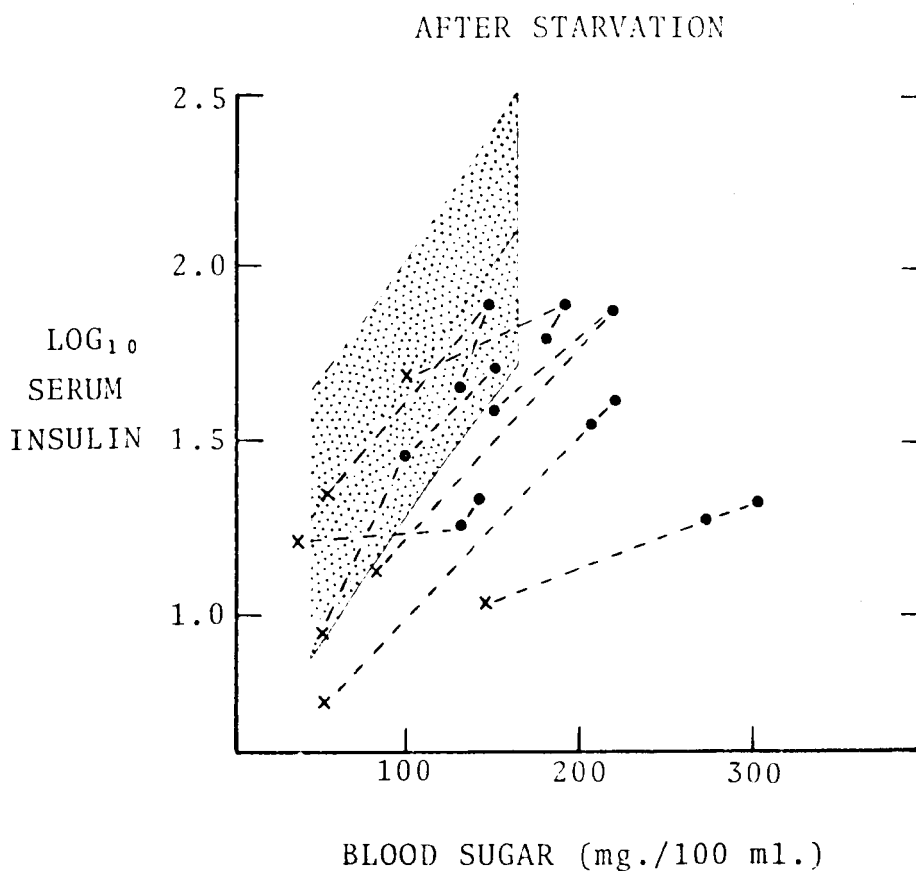


FIGURE 5.

Log₁₀ serum-insulin values of 7 diabetic obese subjects at 0, 60, and 120 minutes of GTT after 7 days total starvation, in comparison with the 95% range of values in 15 non-diabetic obese similarly starved (hatched area). Compare with Figure 2.

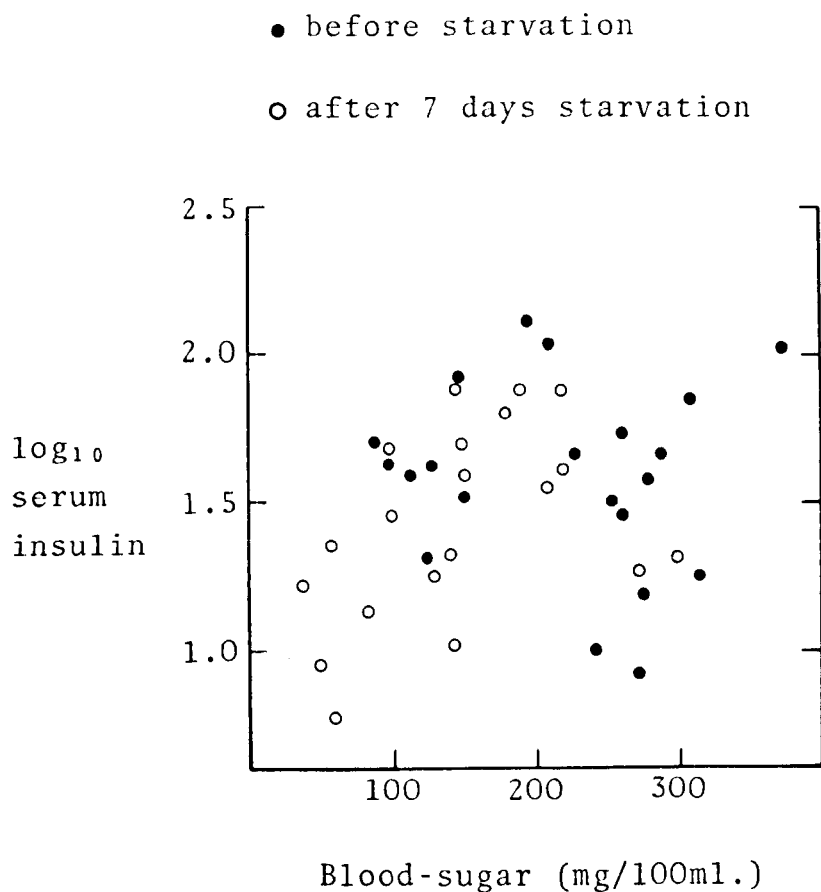


FIGURE 6.

Blood-sugar and $\log_{10}$ serum-insulin levels of 7 diabetic obese subjects before and after total starvation for 7 days. Above a blood-sugar level of 180-200 mg/100ml., insulin values tend to decline.

(Values for the fasting, 60 min., and 120 min. sampling times of the GTT are shown).

●—● BEFORE 7 DAY STARVATION.
 ○---○ AFTER 7 DAY STARVATION.

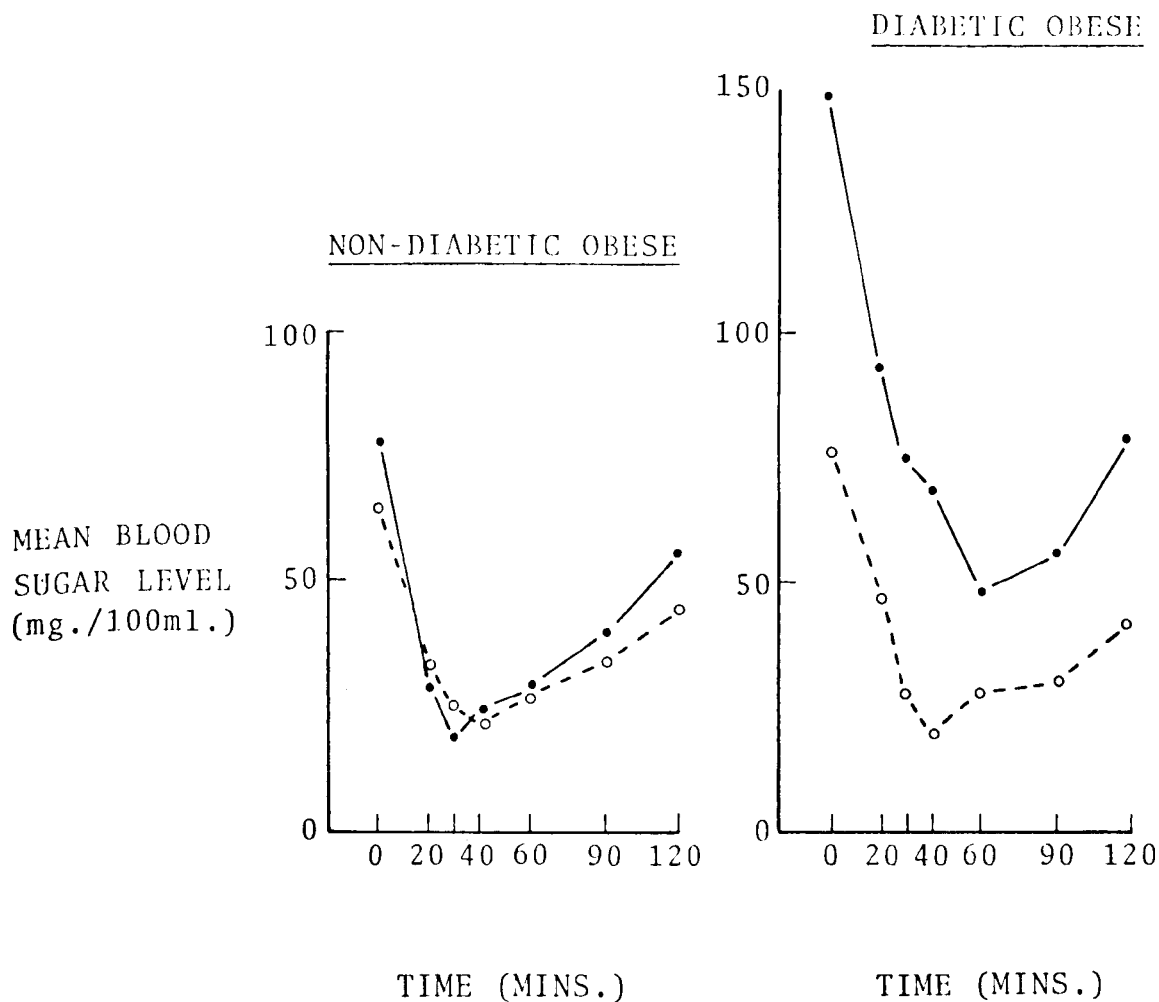


FIGURE 7. Augmented insulin tolerance tests: mean blood-sugar levels in 15 non-diabetic obese and 7 diabetic obese patients before starvation (unbroken lines) and after 7 days total starvation (interrupted lines). No evidence for marked "insulin resistance" is seen after starvation in either the non-diabetic or diabetic subjects.

CHAPTER 6.

SERUM-INSULIN "ONE-HOUR" AFTER GLUCOSE IN A TOTAL NATURAL COMMUNITY

INTRODUCTION

The great variation of insulin levels observed in man has made the task of defining even the more simple relationships with physiological factors a difficult and perplexing one. Attempts, for instance, to relate the recognized deterioration of glucose tolerance that occurs with ageing, to meaningful trends in insulin levels, have given conflicting results. Crockford et alii (1965) found that the serum-insulin response diminished with age in normal volunteers; whereas Chlouverakis et alii (1967) reported a significant rise, with age, of serum-insulin in a normoglycaemic segment of a population survey sample, but this was not found in a further sample of industrial employees (Boyns et alii 1969). Controversy continues as to the exact inter-relationships between elevated insulin levels, obesity, and mild hyperglycaemia (Berson et alii 1965; Karam et alii 1965). A clear need existed for data on even larger series than previously studied. Therefore, in the present study, observations on the levels of insulin were extended to a population survey. The aims may be stated simply: -

(1) To discern the distribution and range of serum-insulin levels in a natural population. In particular, to confirm the log-normal distribution of insulin values, and to assess the evidence for the presence of sub-populations of "high" or "low" insulin secretors.

(2) To define the relationships between serum-insulin and physical and biochemical variables such as age, sex, body size, and the blood-sugar level; and where possible to identify the independent associations of serum-insulin with these factors.

(3) To study serum-insulin in disease states, especially its relevance as a "risk factor" in cardiovascular diseases, and its relationship to diabetes.

Obvious advantages of population studies are the provision of suitably large numbers of subjects for statistical assessment, and the production of information that represents the "natural" condition of man, without the bias inherent in clinical studies in which selection of patients or healthy subjects invariably operates in one way or another. The disadvantages are that control of experimental conditions is much less complete than in the clinical situation, and tests necessarily have to be abbreviated, simple, and acceptable if an adequate population response is to be obtained. "Formal" glucose tolerance tests on an entire community are clearly impractical. Although a glucose load is desirable in order to give a suitable range of both blood-sugar and serum-insulin levels, the variability introduced by such factors as food-ingestion, activity, and the time of day is unavoidable. Nevertheless, attempts can be made to measure some of these factors and thus account for their influence.

The choice of blood sampling one hour after an oral glucose load was made primarily for the convenience of the respondent subjects, and also to achieve optimal punctuality

under the conditions of the study. The available literature (Remein 1961; Hayner 1963; Mitchell and Strauss 1964; Hayner et alii 1965) suggested that this would give quite a good indication of actual glucose tolerance. In addition, the studies of healthy London volunteers (Chapter 3) revealed that at one hour after glucose, the closest association between blood-sugar and serum-insulin levels was obtained. Finally, the "one-hour" blood-sugar level, as used in the Tecumseh population study (Epstein et alii 1965, Ostrander et alii 1965) proved to be a significant risk-factor for coronary heart disease in the initial prevalence study, thus justifying its use for further comparisons.

METHODS

THE POPULATION STUDY.

A comprehensive cross-sectional survey of health and disease was conducted in the Busselton Shire of Western Australia, in November-December, 1966. Purposes of the study included assessment of the range and variation of a large number of clinical and laboratory measurements, and definition of the prevalence of certain common diseases in an Australian community. Busselton was chosen for study because of its stable population, with suitable age structure, and the co-operation and interest of the local authorities. It is a rural area and the town is the centre for surrounding dairy and beef farmers, saw-mill workers, timber getters and titanium miners. The target population was defined from the electoral roll for which registration is compulsory, and approximately 91% of all available adults (21 years of age and over) attended for screening.

Conduct of the survey. Subjects were appointed to attend the Survey Centre at 2-3 minute intervals between 8.30 a.m. and 8.00 p.m. over a two week period. On their appointment cards a specific time was stated for consumption of the glucose load, which was 45 minutes before the stated attendance time. The glucose load consisted of a carbonated lemon flavoured drink containing 50g. of dextrose monohydrate in a volume of 180 ml., prepared by a local factory.

At the Survey Centre, subjects were registered and details of dwelling and occupation obtained. Standard medical questionnaires were administered by doctors, including the recommended questions for angina pectoris and past myocardial infarction (Rose and Blackburn 1966). The stated time of taking the glucose drink was recorded, as was the time of last ingesting food or calorie-containing fluids. Family history of diabetes in first degree relatives was noted. Height without shoes, and weight in minimal summer clothing, was obtained, and the subcutaneous fat-fold of the mid-triceps area measured with Harpenden Calipers (Tanner 1959). Blood pressures were recorded with subjects seated, using a mercury sphygmomanometer, diastolic pressure being the point of disappearance of all sounds. 20 ml. of venous blood were obtained and the time of day documented. Blood-sugar levels were measured immediately by the Autoanalyzer macro-method (Hoffman 1937) and serum was deep-frozen in aliquots for insulin assay and other measurements. Electrocardiograms had been obtained in advance of the survey. The 12-lead tracings were analyzed using the Minnesota Code (Blackburn et alii 1960). The Survey proper, as described above, was conducted over 12 days from November 20th to December 3rd, 1966, with the assistance of a large team of doctors, biochemists and ancillary personnel.

Biochemical analyses were completed within three months in the Biochemistry Department of Royal Perth Hospital, using aliquots of deep-frozen sera. Serum-electrolytes and urea

were estimated by routine Autoanalyzer methods, serum uric acid by the automated procedure of Caraway (1955), serum calcium and magnesium by atomic absorption spectrophotometry, and serum cholesterol by the "direct" method of Watson (1960) which yielded values directly comparable to those obtained by Abell and colleagues' method (1952) with a correlation coefficient of 0.986.

Computation of data was performed on the University of Western Australia's PDP-6 digital computer. "Complete" sets of data from 88% of the target population were transferred to magnetic tape for analysis, after checking and retrieval of omissions. From the data, the following parameters were generated: (1) the "drink-interval" being the interval in minutes from the stated time of consumption of the glucose drink to the recorded time of venepuncture; (2) the "food-interval" from stated time of last ingesting food or calorie-containing fluids to time of venepuncture; (3) the "percentage desirable weight" from the height and weight of subjects as compared to the desirable weights of insured persons (medium frame) in U.S.A. having the lowest mortality in the tables of the Metropolitan Life Insurance Company (Diem 1962).

POPULATION RESPONSE.

The target population comprised 3,719 adults, of whom 3410 (91%) attended the survey; and complete data files, including serum-insulin levels, were obtained for 3313 subjects (88%). The mean interval between the stated time of taking the glucose load and the time of venepuncture (with ± 1 standard deviation) was for males 65 ± 10 minutes, and for females 66 ± 9 minutes. The "one-hour" serum-insulin and the "one hour" blood-sugar levels are thus described in the subsequent account with knowledge of this variability.

IMMUNOASSAY OF SERUM-INSULIN.

The double-antibody technique of Morgan and Lazarow was again used with the modifications described in Chapter 2, but with semi-automation as indicated in Table 1. The automatic aids employed were an "Autodiluter" (Hook and Tucker Ltd.) that drew up 0.1 ml. of unknown samples or standard solutions and dispensed this together with 0.9 ml. of the assay "cocktail" from a reservoir into test-tubes. The assay "cocktail" consisted of standard diluent with Insulin-I¹²⁵ (Amersham) and guinea pig anti-insulin serum (GPAIS, Burroughs Wellcome) at the necessary concentrations (Table 1). It was considered that the 2-4 hours reaction of GPAIS with Insulin-I¹²⁵ in the reservoir during the first dispensing stage would be negligible in view of the 6-day incubation with assay samples to achieve equilibrium conditions. When, however, evidence emerged for within-assay drift (following analysis of the first 8 assays) the GPAIS was later dispensed by a very accurate automatic syringe, the "Repette" (Jencons Co. Ltd), just prior to mixing the contents of assay tubes using a "Vortex" mixer (Labline Instruments Inc.). No suitable automation was devised for the manual decanting stage that separated precipitates from supernatants, which continued to be laborious but technically simple.

The standard insulin used was a preparation of human insulin provided by the Medical Research Council's Division of Biological Standards (London). The crystalline human insulin (reference 66/6) was of stated biological activity 23.5 I U per mg dry weight (confidence limits 22.0 - 25.1 I U per mg dry weight at $p = 0.95$). It was dissolved in a small volume of acidified distilled water (pH 1.6 with hydrochloric acid) and diluted with borate buffer containing 0.2% bovine serum albumin to concentrations of 4000 and 7050 μ U per ml, and stored in aliquots at -20°C .

Standard curves were prepared on each assay day from the deep-frozen aliquots of human insulin. Initially one standard curve per assay was set up, but when evidence for within-assay drift was obtained two or more complete standard curves were employed at regular intervals (every 100 - 150 assay tubes) throughout each assay. Duplicate values for each point on the curves were obtained.

Unknown serum samples were assayed in batches selected randomly. Sera were estimated in duplicate in assays 1 to 20, but single measurements were made for nine out of every ten samples in assays 21 to 27. Variability of duplicates was negligible. Values exceeding 400 $\mu\text{U/ml}$ were assigned a concentration of 400 $\mu\text{U/ml}$ (8 samples).

Computing the assay was essential because of the large number of samples processed. In collaboration (N.S. Stenhouse, Biostatistician) a curve-fitting procedure was devised. When the assay metameter (percentage Insulin-I₁₂₅ bound) was plotted on the Y-axis, and the log-insulin concentration of the standards on the X-axis, a consistently sigmoidal curve resulted (see Chapter 2), and therefore a cubic function of the assay metameter was used to "fit" the points. Also, in order to correct for any systematic "drift" occurring within assays, the tube numbers of points on each standard curve were included as additional variables to be accounted for.

The procedure is best illustrated by an example (assay number 9 of the present series). The logarithmic transformation (X) of the concentrations of the crystalline insulin standards (C) is performed thus: -

$$X = \log_2 (C/50)$$

as shown in Table 2. The assay metameters, "percentage Insulin-I₁₂₅ bound" (p), are listed in Table 3, and give a representative example of a distinct systematic drift occurring between standard curves for the more concentrated insulin

standards. A linear transformation (P) of the metameters (p) is effected by subtracting the approximate value of the median metameter (i.e. for the 50 μ U/ml standard, $p = 41$) and dividing by ten, thus: -

$$P = (p-41) \times 10^{-1}$$

as shown in Table 3. Values are now arrayed round zero, facilitating more accurate linear regression analysis and giving more equal weight to values at the extremes of the curves.

Multiple regression analysis is then performed (Stenhouse 1965) with the log-concentrations of the insulin standards (X) as the dependent variable; and as determining variables, the following functions of the linear transform of assay metameter (P) and the assay Tube number (T):

(i)	P	(iv)	T
(ii)	P x P	(v)	T x P
(iii)	P x P x P		

The square and the cube of (P) are included in order to "fit" the sigmoidal curve, and (T) and (T x P) to account for within-assay drift. Results of the multiple regression analysis for this example are shown in Table 4. The form of the regression equation derived is: -

$$X=Y+(b_1 \times P)+(b_2 \times P^2)+(b_3 \times P^3)+(b_4 \times T)+(b_5 \times P \times T)$$

Where Y is the intercept on the Y axis and $b_1 \dots b_5$ the regression coefficients for (P)....(P x T) respectively. In this example, the contribution of each determining variable is highly significant (Table 4) and an extremely close fit for the curves is obtained, as indicated by the coefficient of multiple correlation ($R = 0.9997$) and the "unbiased" index of determination ($A = 0.994$). In essence, this procedure converts the sigmoidal curves into straight lines with the standards at equidistant points. Thus, with knowledge of the assay metameter (P) and the tube number (T), the insulin concentrations of unknown samples can be accurately calculated from the equation,

bearing in mind that the close fit obtained is with reference to the logarithmic concentrations of standard insulin. Table 5 shows the results of "playing back" the equation, for the example described, to give the calculated concentrations of the insulin standards in absolute insulin levels ($\mu\text{U}/\text{ml.}$) These are very close to the expected concentrations, and the "drift" within assay is substantially corrected.

The performance of the 27 assays used is shown in Table 6, which gives the values for "A", the "unbiased" index of determination, to indicate the precision of standard curve fitting, which was uniformly high. All standard curves were also checked visually: the "sensitivities" almost invariably were less than $6.3 \mu\text{U}$ per ml. The mean values, as calculated by the curve-fitting procedure for the crystalline insulin standards is shown in Table 7. These matched the expected absolute values; and the range of standard deviations gives an indication of the overall accuracy and sensitivity of assays plus computing. Unknown samples in random batches were estimated in duplicate in assays 1 to 20, but single measurements only were made for nine out of every ten samples in assays 21 to 27. Variability of duplicates was negligible.

Quality control procedures (Table 6) included the estimation of aliquots of two standard sera ("A" and "B" for assays 1 to 20; "C" and "D" for assays 20 to 27) which were generally measured in conjunction with standard curves at the beginning, the middle, and the end of each assay. Within-assay drift was evident (Table 6) in some of the early assays and persisted even when more than one standard curve per assay was routinely employed. Retrospective quality control checks included use of the $\log_{10}$ insulin values obtained for the unknown samples and the degree of correlation found with (i) their corresponding blood-sugar levels, and (ii) their assay tube numbers. Correlation coefficients for $\log_{10}$ insulin with blood-sugar were positive and invariably significant, providing

the reassurance that no substantial errors of technique or of sample identification had occurred, and indicating the "biological" validity of each assay. In 5 assays, there were significant correlations between $\log_{10}$ insulin and the assay tube number, suggesting a systematic within-assay drift that did not always correspond to the trend observed in the standard sera (Table 6). No explanation was found for this phenomenon in two of the assays (numbers 16 and 21) which involved small numbers of samples, but in the remaining 3, correlation coefficients of the same order were obtained between assay tube number and the blood-sugar level, thus chance probably accounted for the trend of insulin values discerned.

The standard sera gave values that varied considerably both within and between assays. To provide an indication of between assay variability, results from the first set of sera in each assay were analyzed, the coefficient of variation ranging from 10.8 to 14.1% using absolute insulin values, although the variability was much less in terms of $\log_{10}$ insulin, as shown in Table 8. When plotted on a quality control chart (Figure 1) the systematic nature of the between assay variability is seen, since both standard sera and the log-means of the unknown samples showed striking parallel trends. Analysis of variance of all $\log_{10}$ insulin values obtained for the 4 standard sera is presented in Table 9 and indicates that between-assay variability was significantly greater than within-assay variability for each serum standard.

The cause of variability of the assay was not established. Suggestive evidence emerged on subsequent analysis that the slope of the standard curves, and the extent of combination of Insulin-I¹²⁵ with antibody would account for more than half of the variation observed in the serum standards. It is possible, therefore, that variable integrity of the labelled hormone was responsible for the systematic between-

assay trends, particularly since attempts were made to maintain strict uniformity of technique throughout the series of assays. The topic was not further pursued. The assay values for unknown samples were all "accepted", but with clear knowledge of the variability of the technique as described.

RESULTS.

These are described and discussed in two sections:

- (1) The "one-hour" serum-insulin and its relationship to physiological variables.
- (11) The "one-hour" serum-insulin in health and disease.

TABLE 1. FLOWSHEET FOR THE SEMI AUTOMATED IMMUNOASSAY OF INSULIN USED IN THE POPULATION STUDIES.

(Standard diluent: borate buffer with 0.2% bovine albumin (v) pH 8.5)

FIRST ANTIBODY REACTION	0.1 ml. Standard human insulin or serum sample	
	+ 0.9 ml. ¹²⁵ I-insulin $\approx$ 3 μ IU	Auto-diluter
	+ 0.1 ml. Guinea-pig anti-insulin serum 1:16000	Automatic pipette
SECOND ANTIBODY REACTION	* Mix	Vortex Mixer
	* Incubate at 4°C for 6 days	
	+ 0.1 ml. Guinea-pig serum $\sim$ 1:100 with heparin (30 IU)	Automatic pipette
	+ 0.1 ml. Rabbit anti-guinea pig γ globulin $\sim$ 1:4	
	* Mix	
	* Incubate at 4°C for 24 hours	
	* Centrifuge at 4°C for 20 mins.	
	* Decant supernatant	
	* Count precipitate and supernatant tubes	Automatic well counter
	* Compute assay response metameter (percent. counts bound)	

TABLE 2.

Concentrations of crystalline insulin standards used, and the logarithmic transformation employed for curve-fitting.

ASSAYS 1 - 16

CONCENTRATIONS (C) OF CRYSTALLINE INSULIN STANDARDS		LOGARITHMIC TRANSFORMATION (X): $X = \log_2 (C/50)$
6.25	$\mu\text{U/ml.}$	- 3.0
12.5	"	- 2.0
25	"	- 1.0
50	"	0.0
100	"	+ 1.0
200	"	+ 2.0
400	"	+ 3.0

ASSAYS 17 - 27

CONCENTRATION (C) OF CRYSTALLINE INSULIN STANDARDS		LOGARITHMIC TRANSFORMATION (X): $X = \log_2 (C/44)$
5.5	$\mu\text{U/ml}$	- 3.0
11	"	- 2.0
22	"	- 1.0
44	"	0.0
88	"	+ 1.0
176	"	+ 2.0
352	"	+ 3.0

TABLE 3. Data from Sample Assay (No.9) showing the response metameter (percent. counts bound) for 3 standard curves through the Assay, and the linear transformation employed for curve-fitting.

CONCENTRATION OF CRYSTALLINE INSULIN STANDARDS	ASSAY METAMETER (p) percent. counts bound			LINEAR TRANSFORMATION (P) OF ASSAY ₁ METAMETER: $P = (p - 41) \times 10^{-1}$		
	Tube No.	Tube No.	Tube No.	Tube No.	Tube No.	Tube No.
	1 - 14	101-114	201-214	1-14	101-114	201-214
6.25 μ U/ml.	60.6%	60.6%	60.6%	-1.96	-1.96	-1.96
12.5 "	55.6%	55.7%	55.9%	-1.46	-1.47	-0.85
25 "	48.3%	49.5%	49.5%	-0.73	-0.85	-0.85
50 "	40.5%	41.6%	42.6%	+0.05	-0.06	-0.16
100 "	33.1%	34.4%	35.3%	+0.79	+0.66	+0.57
200 "	27.0%	28.5%	29.7%	+1.40	+1.25	+1.13
400 "	22.4%	24.2%	26.1%	+1.86	+1.68	+1.49

TABLE 4. Multiple Regression analysis of factors determining the log-concentration (X) of the insulin standards, from the data of Assay No.9.

The determining variables used are functions of P (the linear transform of the assay response metameter) and T (the tube number) as shown in Table 3.

DETERMINING VARIABLES	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE (and significance)
P	- 11.67	33.8 ($p < 0.001$)
P x P	+ 7.100	6.4 ($p < 0.001$)
P x P x P	- 108.4	10.3 ($p < 0.001$)
T	+ 1.790×10^{-3}	10.5 ($p < 0.001$)
T x P	- 1.091×10^{-2}	8.1 ($p < 0.001$)
- - - - -	- - - - -	- - - - -

Intercept on Y axis = - 0.1224

Coefficient of multiple correlation (R) = 0.9997

"Unbiased" index of determination (A) = 0.9994

TABLE 5. Results from the Sample Assay (Table 3) showing the calculated concentration of Crystalline Insulin Standards as derived from the linear Multiple Regression Equation (Table 4).

EXPECTED CONCENTRATION OF CRYSTALLINE INSULIN STANDARDS	CALCULATED CONCENTRATION OF CRYSTALLINE INSULIN STANDARDS (μ U/ml.)		
	Tube No. 1-14	Tube No. 101-114	Tube No. 201-214
6.25 μ U/ml. :	6.5	6.3	6.2
12.5 "	12.4	12.4	12.2
25 "	25.5	24.2	25.8
50 "	48.8	49.6	50.8
100 "	95.8	98.6	106.5
200 "	201.8	201.4	211.6
400 "	420.6	391.7	365.6

TABLE 6.

Summary of the performance of 27 assays in which 3400 unknown serum samples were measured: Quality control data includes (1) the "unbiased index of determination" to assess the precision of standard curve-fitting; (2) two standard sera assayed within and between assay, for which all values are shown; (3) correlation coefficients obtained between the $\log_{10}$ insulin values of unknown serum samples and their corresponding blood-sugar levels and assay tube numbers.

TABLE 6. (Continued)

ASSAY	STANDARD CURVES		SERUM SAMPLES		STANDARD SERA	
	No. in each Assay	"Unbiased" index of determination 'A'	Number in ea. Assay	Correlation Coefficients for Log_{10} Insulin levels with Blood-sugar (mg/100ml)	Assay Tube Number	Insulin levels ($\mu\text{U/ml}$) obtained for Sera:
						A: B:
1	1	0.998	96	0.27 ($p < .01$)	+0.03	62, 47
2	1	0.998	151	0.38 ($p < .001$)	-0.10	79
3	1	0.997	209	0.22 ($p < .001$)	+0.10	69, 71
4	1	0.999	134	0.41 ($p < .001$)	-0.10	67
5	1	0.997	155	0.40 ($p < .001$)	+0.19 ($p < .05$) ¹	70, 46
6	1	0.999	93	0.31 ($p < .01$)	-0.07	78
7	1	0.998	90	0.43 ($p < .001$)	-0.13	73, 65
8	1	0.987	134	0.31 ($p < .001$)	-0.02	76, 53
9	3	0.999	117	0.23 ($p < .01$)	+0.12	64, 73
10	2	0.998	109	0.20 ($p < .05$)	+0.10	71, 69, 73
11	2	0.998	100	0.38 ($p < .001$)	-0.14	30, 32, 34
12	3	0.990	129	0.40 ($p < .001$)	+0.06	69, 72, 73
13	3	0.998	106	0.27 ($p < .01$)	+0.04	97, 85
14	2	0.999	83	0.39 ($p < .001$)	-0.14	81, 65
15	2	0.998	97	0.33 ($p < .001$)	-0.14	71, 85
16	2	0.998	47	0.29 ($p < .01$)	+0.34 ($p < .01$)	75, 79
17	3	0.996	139	0.36 ($p < .001$)	-0.01	102
18	3	0.993	140	0.28 ($p < .001$)	-0.01	78, 91, 74
19	3	0.998	178	0.44 ($p < .001$)	-0.08	63, 82, 77
20	3	0.996	177	0.36 ($p < .001$)	-0.05	76, 78, 79
21	1	0.994	79	0.49 ($p < .001$)	+0.31 ($p < .01$)	(83) (C: 45, 46, 50) * (D: 35, 35, 35)
22	3	0.990	179	0.46 ($p < .001$)	-0.14	54
23	3	0.995	152	0.39 ($p < .001$)	+0.17 ($p < .05$) ²	46, 53, 35
24	4	0.996	181	0.19 ($p < .01$)	-0.15 ($p < .05$) ³	59, 58, 47
25	3	0.998	150	0.18 ($p < .001$)	+0.07	47, 42, 44, 40.
26	2	0.991	75	0.29 ($p < .05$)	+0.12	47, 42, 48
27	2	0.996	72	0.24 ($p < .05$)	-0.03	39, 37, 37
						45, 46, 45

* New serum standards (C and D)

TABLE 7. Summary of absolute values obtained for crystalline insulin standards by the computed curve-fitting procedure in 27 assays.

<u>CONCENTRATION OF INSULIN STANDARDS (μU/ml)</u>					
ASSAYS 1 - 16			ASSAYS 17 - 27		
EXPECTED VALUES	COMPUTED VALUES Mean $\pm$ S.D. (V%)		EXPECTED VALUES	COMPUTED VALUES Mean $\pm$ S.D.	
6.25	6.41	$\pm$ 0.45 (7%)	5.5	5.75	$\pm$ 0.49 (8%)
12.5	12.16	$\pm$ 0.65 (5%)	11	10.39	$\pm$ 0.76 (7%)
25	25.4	$\pm$ 2.4 (9%)	22	22.3	$\pm$ 1.5 (7%)
50	50.5	$\pm$ 3.3 (7%)	44	44.8	$\pm$ 2.4 (5%)
100	100.2	$\pm$ 3.8 (4%)	88	88.5	$\pm$ 6.1 (7%)
200	201.3	$\pm$ 14.4 (7%)	176	175.7	$\pm$ 17.4 (10%)
400	396.4	$\pm$ 28.8 (7%)	353	348.6	$\pm$ 33.6 (10%)

S.D. = Standard deviation.

V% = Coefficient of variation.

TABLE 8. Mean values obtained for 4 Standard Sera, and the variation observed "between-assay". The first standard samples only of each assay have been used.

STANDARD SERA	ABSOLUTE INSULIN VALUES (μ U/ml)			LOG ₁₀ INSULIN VALUES		
	Mean [±]	S.D.	Coefficient of variation	Mean	[±] S.D.	Coefficient of variation
"A"	75	[±] 10.1	13.5%	1.87	[±] 0.055	3.0%
"B"	34	[±] 4.7	14.1%	1.52	[±] 0.058	3.8%
"C"	48	[±] 5.7	11.9%	1.70	[±] 0.049	2.9%
"D"	33	[±] 3.6	10.8%	1.52	[±] 0.049	3.2%

TABLE 9. Standard analysis of variance of all $\log_{10}$ insulin values obtained for standard sera (the absolute values are shown in Table 6.).

ANALYSIS OF VARIANCE ($\log_{10}$ insulin values for standard sera).

SOURCE OF VARIATION	D.F.	S.S.	M.S.	CO-EFFICIENT OF VARIATION	VARIANCE RATIO.
<u>SERUM "A":</u>					
Between assays:	19	0.1257	0.00662	(4.4%)	2.17 (p<0.05)
Within assays:	20	0.0609	0.00304	(2.9%)	
Total :	39	0.1866			
<u>SERUM "B":</u>					
Between assays:	19	0.1462	0.00769	(5.8%)	7.78 (p<0.01)
Within assays:	22	0.0217	0.00098	(2.1%)	
Total :	41	0.1679			
<u>SERUM "C":</u>					
Between assays:	7	0.0351	0.00501	(4.7%)	7.96 (p<0.01)
Within assays:	15	0.0094	0.00063	(1.7%)	
Total :	22	0.0445			
<u>SERUM "D":</u>					
Between assays:	7	0.0470	0.00671	(5.0%)	3.47 (p<0.05)
Within assays:	15	0.0290	0.00194	(2.7%)	
Total :	22	0.0760			

D.F. = degree of freedom

S.S. = sums of squares

M.S. = mean square

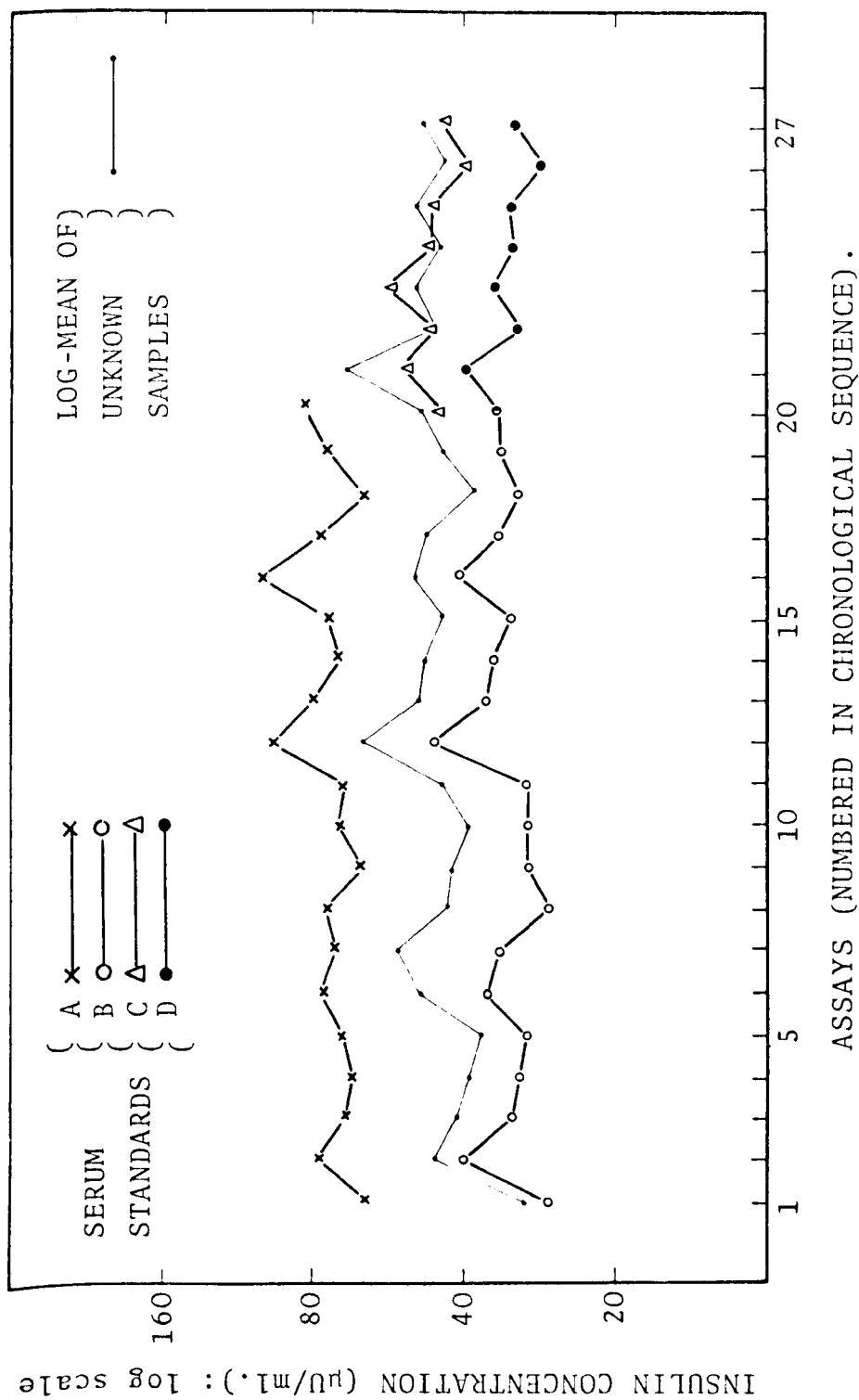


FIGURE 1.

QUALITY CONTROL CHART, showing the values obtained for the first estimate of serum standards in each assay, and the log-mean of unknown samples assayed.

RESULTS

I. THE "ONE-HOUR" SERUM-INSULIN AND ITS RELATIONSHIP TO PHYSIOLOGICAL VARIABLES.

DISTRIBUTION AND RANGE OF SERUM-INSULIN VALUES.

The distributions of the absolute insulin values obtained in 1629 males and 1684 females approximately one hour after 50g. glucose orally are shown in Figure 2 and Table 10. The distributions are unimodal and positively skewed, and differ profoundly from the expected normal distribution in terms of the numbers of subjects delineated by 1 and 2 standard deviations from the mean ($p < 0.001$.)

Logarithmic conversion of the insulin levels yielded corresponding values for means and medians, and the standard deviations of the $\log_{10}$ serum-insulin concentrations segregated close to the expected proportion of subjects (Table 11). No significant difference from the normal distribution was found by the chi-squared test. Figure 3 shows the results of plotting the percentiles of $\log_{10}$ serum-insulin on linear probability paper. The small departures from linearity below the 2nd percentile and above the 98th percentile values do not provide compelling evidence for separate populations of "high" or "low" insulin secretors. Within age groups, the closeness of fit of the $\log_{10}$ insulin values with the normal distribution continues, as shown in Figure 4.

The overall mean levels, and the range of ± 2 standard deviations, for $\log_{10}$ serum-insulin (with corresponding absolute values in $\mu\text{U/ml.}$) were:

	<u>Mean $\log_{10}$ serum-insulin</u>	<u>Range of mean $\pm$ 2 SDs</u>
1629 males	$1.625 \pm 0.279 (42 \mu\text{U/ml} \times)$	1.07-2.18 (12-152 $\mu\text{U/ml} \times$)
1684 females	$1.686 \pm 0.262 (49 \mu\text{U/ml} \times)$	1.16-2.21 (15-162 $\mu\text{U/ml} \times$)

EFFECTS OF AGE AND SEX.

"One-hour" $\log_{10}$ serum-insulin values by sex and 10-year age groups are shown in Figure 5 and Table 12. At all ages, females have significantly higher mean values than males. In males, serum-insulin rises progressively from 21-29 years onwards, but an increase with age in females is not evident until 50 years, when a trend parallel to that of males occurs. The tenth percentile ranges for $\log_{10}$ serum-insulin in males and females by 10-year age groups are graphed in Figure 6. The rise of insulin values with age is "global", occurring to an equal extent in the lower, middle, and upper ranges of both sexes. The very wide range of insulin values is clearly demonstrated, as is the symmetrical distribution of the $\log_{10}$ values about the mean, for each age group.

The relationship between serum-insulin and age is not simple, since other factors related to insulin levels are involved. Table 13 shows that the mean blood-sugar levels at "one-hour" also rise steeply with age, but do not differ greatly between the sexes, although tending to be higher in the older females. The degree of obesity, expressed as percentage desirable weight or measured as the mid-triceps fat-fold thickness, also tends to increase as age progresses.

RELATIONSHIP OF SERUM-INSULIN TO PHYSIOLOGICAL VARIABLES.

The large number of physical and biochemical variables measured in all subjects are listed in Table 14, with the mean values, the standard deviations, and the absolute ranges for males and females. The units stated for each variable are those

used in the subsequent tabulations.

The simple correlations that obtained between these variables and the "one-hour" $\log_{10}$ serum-insulin are shown in Table 15. Many of the variables had very highly significant correlation coefficients with insulin of which the most powerful were the positive associations of the blood-sugar level, age, and percentage desirable weight. Other indices of obesity that showed lesser correlation coefficients with insulin values were mid-triceps fat-fold and actual body weight ($p < 0.001$). Height had a highly significant negative correlation with $\log_{10}$ serum-insulin ($p < 0.001$). Blood-pressure showed a positive association, particularly the systolic ($p < 0.001$) and to a lesser extent the diastolic pressure ($p < 0.01$). In both sexes, the "food-interval" was negatively related to insulin ($p < 0.001$); but in males only the "drink-interval" ($p < 0.001$) and the actual time of day ($p < 0.05$).

Of the biochemical variables, serum uric acid and to a lesser extent the serum cholesterol ($p < 0.001$) had positive correlation coefficients, but the remaining associations do not achieve corresponding significance in males and females. Thus although there is a general tendency for the levels of all serum electrolytes to have negative correlations with the $\log_{10}$ insulin concentration, only those of serum potassium ($p < 0.05$) and magnesium ($p < 0.05$) in males, and serum chloride ($p < 0.01$) and bicarbonate ($p < 0.05$) in females, are statistically significant. The serum urea in females is positively related to $\log_{10}$ serum-insulin ($p < 0.01$).

These data demonstrate the overall relationships that exist with the "one-hour" $\log_{10}$ serum-insulin, but give no indication of the independent contribution of various factors. It is important to ascertain, for example, whether the positive correlation of insulin with age represents a direct effect or one mediated by the common association of these

variables with the blood-sugar levels, which rise steeply with age. That intricate interrelationships exist is demonstrated in Table 16, where it is shown that $\log_{10}$ serum-insulin, blood-sugar, percentage desirable weight and age are each associated with the other variables at a high level of statistical significance. Before proceeding with multiple regression analysis to determine the independent contribution of these variables, the nature of the simple associations was examined, and the extent of some of the inter-relationships ascertained by plotting the means and ranges of serum-insulin by various class intervals of other factors.

SERUM-INSULIN AND BLOOD-SUGAR LEVELS.

The mean "one-hour" $\log_{10}$ serum-insulin, together with the ranges of ± 1 standard deviation, for 50 mg/100ml classes of blood-sugar (less than 50 mg/100 ml, 50-99, 100-149, 150-199 mg/100ml, and greater than 200 mg/100ml) are depicted in Figure 7. The curve of $\log_{10}$ serum-insulin rose steeply with increasing blood-sugar, but tended to flatten or even fall with blood-sugar levels in the higher ranges. The figure also shows that subjects with higher blood-sugar levels were older and more obese, and showed a wider scatter of insulin values.

SERUM-INSULIN AND OBESITY.

The effect of obesity, expressed as percentage desirable weight, on $\log_{10}$ serum-insulin is shown in Figure 8. In males, a very consistent linear relationship is apparent from the very thin (body-weight less than 70%) to the grossly obese (body-weight greater than 160%) with a uniform range of insulin values for a given percentage desirable weight. In females, no effect of obesity was discerned until percentage desirable weight exceeded 120%, when a progressive rise of serum-insulin occurred, but less steep than for males. More-or-less similar trends were evident with the mid-triceps fat-fold

thickness, which provides a direct measurement of adipose tissue, but is a crude index of total obesity (Figure 9). Obesity, thus assessed, has a more profound influence on insulin levels in males than in females, in part because the more lean females exhibit a "plateau" of serum-insulin values.

INTER-RELATIONSHIPS OF SERUM-INSULIN WITH BLOOD-SUGAR, AGE, AND OBESITY.

The interaction of age and obesity with insulin values was examined by ascertaining the mean $\log_{10}$ serum-insulin of subjects in the upper and lower quintiles (age and sex-specific) of percentage desirable weight in comparison with the overall mean values, as shown in Figure 10. Obesity and age have a cumulative effect on the "one-hour" serum-insulin of males, for the obese males (body-weight greater than 119-129%) show a rise of insulin levels from the age range 21-29 years onwards, whereas the levels of the lean males (body-weight less than 97-102%) do not rise until the age of 50-59 years. In females, obesity has a much smaller effect and similar trends, although present are less clearly seen.

The available numbers of subjects within each decade allow one further subdivision of the data, so that the separate influence of blood-sugar levels in addition to those of obesity and age can be visualized. The age-specific and sex-specific 80th percentile values of percentage desirable weight and blood-sugar were used as the cutting points to divide "upper" (U) from "lower" (L) ranges. The mean $\log_{10}$ serum-insulin has been calculated for subjects having both variables in the lower range (LL), one variable in the upper range (LU or UL), and both variables in the upper range (UU). The proportion of subjects segregated by these divisions (63%, 16%, 16% and 5% respectively) was identical in males and females and is close to the expected proportion. The results are shown in Figure 11. In males, coexistence of obesity and hyperglycaemia produced the highest mean insulin values; and "upper range" percentage

desirable weight exerted a more profound influence in elevating serum-insulin than "upper range" blood-sugar. Age appears to have exerted an effect prematurely on males with high body-weight and/or blood-sugar; in contrast, males having both variables in the "lower range" show no effect of age on serum-insulin until 60-69 years. In females, less clear trends existed, but the magnitude of the effects of obesity alone or of hyperglycaemia alone appeared to be equivalent, and the influence of age was more uniform. From this comparatively crude analysis, it might be suggested that in both sexes blood-sugar and obesity exerted an independent influence on the elevation of serum-insulin, and that the combination of factors appeared to be cumulative. In males only, both of these factors "accelerated" the rise of insulin values with age.

EFFECTS OF THE "DRINK-INTERVAL", THE "FOOD-INTERVAL", AND THE TIME OF DAY.

Inter-relationships between the interval from the glucose load, the interval from last taking food or calorie-containing fluids, and the time of day, were sufficiently complex as to defy simple interpretations of their relationship with the blood-sugar and serum-insulin levels (Table 17). In males, a significant negative correlation existed between the "drink-interval" and both $\log_{10}$ serum-insulin ($p < 0.001$) and blood-sugar ($p < 0.01$), but not in females. The extent of the effect of the "drink-interval" on $\log_{10}$ serum-insulin is shown in Figure 12. Males showed a regular decline of mean $\log_{10}$ serum-insulin from 30 to 90 minutes after glucose, whereas the similar but slight relationship in females was evident only between 30 and 45 minutes. In both sexes, the "food-interval" was positively correlated with blood-sugar values ($p < 0.01$) but negatively with serum-insulin ($p < 0.001$) as illustrated in Figure 13. Although the time of day (8.30 a.m. to 8.00 p.m.) showed no strong linear correlation with insulin values, a definite relationship existed, The fluctuations of serum-insulin /as shown in Figure 14.

observed did not indicate an innate circadian rhythm but rather reflected the very close inverse association between insulin levels and the "food-interval", which is also shown. The tides of insulin related clearly to conventional meal-times. In contrast, the mean blood-sugar levels observed throughout the day revealed possible diurnal variation, rising to a mean peak in the mid-afternoon.

FACTORS DETERMINING THE "ONE-HOUR" SERUM-INSULIN BY MULTIPLE REGRESSION ANALYSIS.

Multiple regression analysis provides one of the most suitable and elegant techniques for assessing the independent contribution of variables associated with serum-insulin. All of the physiological factors measured in the study (see Table 14) were included as possible determining variables, together with the square and cube of each to allow for the possibility of significant non-linear relationships. The final equations obtained for the "prediction" of $\log_{10}$ serum-insulin are shown for males and females in Table 18. The significance attributed to each variable, by Student's "T" value gives an indication of the strength of the association with $\log_{10}$ serum-insulin. Only three variables, age, the blood-sugar, and the "food-interval" had non-linear relationships of statistical significance.

Blood-sugar levels were the major determinants of serum-insulin. The partial regression of $\log_{10}$ serum-insulin on blood-sugar levels for males and females is shown in Figure 15. Mean serum-insulin levels rose with increasing blood-sugar in an almost linear fashion over the range 25 to 180 mg/100ml. but at blood-sugar levels greater than 180 mg/100ml. there was a progressive fall-off in mean serum-insulin. Although only a small number of subjects (35 males, 32 females - see Figure 7) had blood-sugar levels greater than 200 mg/100ml, the contribution of the latter was important, as seen by the significance of the regression coefficients for (blood-sugar)²

and (blood-sugar)³ in Table 18. The validity of this finding is supported by the consistent and corresponding trends that occurred in both males and females.

Age exerted an effect independent of the blood-sugar and of other factors (Figure 15) which conformed fairly closely to the simple relationship (Figure 5). Above the age of 40 years in males, and 50 years in females, a moderate rise of the "one-hour" serum-insulin occurred as age progressed.

Height had a highly significant negative association, and actual body weight an even greater positive association, with $\log_{10}$ serum-insulin, the effect of both being more marked in males than in females (Figure 15). These variables were preferred by the analysis over percentage desirable weight and fat-fold as having the greater independent contributions. The relationships are linear.

In males, the negative effect of the "drink-interval" was significant (Figure 16) and was similar to the observed relationship (Figure 12). A more substantial independent effect of the "food-interval" on serum-insulin is seen by multiple regression analysis in Figure 16, than in the crude data (Figure 13). Figure 16 shows also the independent association of the "food-interval" with blood-sugar, from a separate analysis. A paradoxical relationship existed; if the "one-hour" blood sample was obtained within 3 hours of taking food, comparatively high serum-insulin and low blood-sugar levels were found. Between 3 and 8 hours after food, the general trends reversed, so that blood-sugar levels exceeded, and serum-insulin levels were lower than the overall mean values. Beyond 8 hours, there were too few subjects contributing to the curves for valid interpretation. The time of day did not contribute as a significant independent variable.

Of the biochemical determining variables other than blood-sugar, serum-uric acid and $\log_{10}$ serum-insulin continued to show a highly significant association in both sexes, although

age and body size were accounted for. A negative relationship of serum potassium with insulin in males was confirmed ($p < 0.001$) and this emerged also in females ($p < 0.05$). The remaining negative associations were with serum urea in males ($p < 0.001$) and with serum bicarbonate in females ($p < 0.001$).

It is important perhaps to mention those variables excluded by the analysis. Systolic and diastolic blood-pressure and serum cholesterol formerly showed significant simple correlations (Table 13) but exerted no independent effect on $\log_{10}$ serum-insulin, thus the relationship observed was mediated by the effect of other variables.

The coefficients of multiple correlation are 0.5890 for males, and 0.5346 for females. The proportion of the variability of $\log_{10}$ serum-insulin accounted for by the regression equation is 34% for males and 28% for females.

THE POSSIBLE EFFECT OF OSMOLALITY.

On the basis of clinical suspicion that hyperosmolar conditions are sometimes associated with deficient insulin secretion (to be discussed) a figure for plasma osmolality was derived from the data. The levels of blood-sugar and serum urea, and the concentrations of all serum electrolytes measured (Table 14) were used in a formula that provides a fairly reliable estimate of the true plasma osmolality (Advanced Osmometer Manual 1964). This variable was then included in a further multiple regression analysis of factors determining the "one hour" serum-insulin.

In females, plasma osmolality was found to have an independent negative association with $\log_{10}$ serum-insulin ($p < 0.001$). In the same equation the nature of the independent relationship of blood-sugar with $\log_{10}$ serum-insulin was modified by the inclusion of osmolality but only for the higher ranges of blood-sugar levels, as shown in Figure 17.

It would appear that part of the negative "effect" of blood-sugar levels exceeding 180 mg/100 ml on the serum-insulin values is accounted for by the estimate of plasma osmolality. Although the degree of hyperosmolality was mild, the association with lower insulin levels was highly significant. No such a relationship was found for males.

DISCUSSION

The major advantage of this form of study has been the confident identification of some physiological factors associated with the "one hour" serum-insulin level, and delineation of the nature of their independent relationships. With the large numbers studied, the wide range and profound skewness of distribution of insulin levels has again been demonstrated; but clear confirmation has been obtained that logarithmic transformation renders the distribution symmetrical and statistically normal. Use of the $\log_{10}$ values is recommended for the practical purpose of depicting meaningful ranges (standard deviations, standard errors of the mean), as for example when graphs are used to summarize data from large samples. Also "normalized" data is to be preferred for statistical comparisons particularly when variables are as markedly non-normal as absolute serum-insulin values. The distribution of serum-insulin was unimodal and no evidence emerged from this study for the existence of distinct sub-populations of high or low insulin secretors. Such evidence was sought because of suggestions that diabetes-prone individuals could be discriminated by a distinct increase in synalbumin antagonism (Vallance-Owen 1966) or alternatively by a characteristically low insulin response to glucose infusion (Cerasi and Luft 1963). It must be acknowledged, however, that the uncontrolled aspects of the survey could have caused sufficient variability to mask hints of bimodality.

It is indeed recognized that the study lacked the standardized conditions usually observed in formal clinical tests; and various factors amenable to better control in future studies deserve consideration. In males, serum-insulin showed a moderate decline with the "drink-interval". In both sexes, the "food-interval" was found to exert some considerable effect on both blood-sugar and serum-insulin levels. Subjects tested within 3 hours of taking food showed higher serum-insulin but lower blood-sugar levels. The improved glucose tolerance reflects the "Staub-Traugott" effect, which has been studied in some detail (Somersalo 1950). In summary, a second glucose load administered within 1-2 hours of an initial oral load results in a less marked elevation of blood-sugar levels. The mechanism, which is not yet precisely explained, may depend on the priming effect of the various intestinal factors (secretin, gastrin, "gut-glucagon") that provide an augmented insulin response to the second glucose load. In addition the suppression of growth hormone secretion by prior food ingestion may result in improved insulin sensitivity. Conversely, in the absence of food intake within 3 hours, insulin secretion may be less through the lack of a priming effect, and post-absorptive rises in growth hormone are likely to contribute to insulin resistance, as has been recently demonstrated (Yalow et alii 1969). Although the time of day (between 8.30 a.m. and 8 p.m.) showed a significant influence on the mean "one hour" blood-sugar levels, and a characteristic pattern of diurnal variation with a peak level at about 3 p.m. was evident (Figure 14), the fluctuations of serum-insulin were closely related to the food-interval, and multiple regression analysis revealed no independent contribution of the time of day to insulin levels. Diurnal variation of glucose tolerance has been documented (Jarrett and Keen 1969) with higher blood-sugar levels in afternoon compared with

morning tests, but the physiological factors responsible have not been identified. Varying sensitivity of pancreatic beta cells to a glucose stimulus has not been identified in the present study.

The size of the glucose load administered influences the observed insulin response. This is inferred from the negative relationship of height to serum-insulin levels. Height was shown to have an association independent of body weight and other factors and therefore indicates non-adipose body mass. A standard glucose load, administered to subjects of varying size is, in fact, a variable stimulus. When comparing groups of subjects with substantial differences in size, a glucose load proportional to an index of lean body mass (such as height) should be given, although for survey purposes a uniform load is obviously more convenient. A further source of variability of insulin levels may well have been the quantity of food ingested in proximity to the glucose load: this could not conveniently be ascertained.

The final uncontrolled factor before and during the test was physical activity. The elderly, or the obese, or the diseased, may have exerted themselves less during the test, and thus exhibited higher blood-sugar levels than their more active counterparts. However the available evidence (Nikkila et alii 1968; Schalch 1967) indicates that lower blood-sugar levels occur during exercise without marked change in the insulin levels, although a slight but non-significant tendency existed for serum-insulin to fall.

It may be said, in defence of this form of study, that many of the sources of uncontrolled variability are likely to have been randomly distributed (such as variations in the time intervals described); and that factors likely to contribute systematic bias to biochemical measurements (such as the degree of physical activity, or the quantity

of food ingested in different age groups or occupational categories) will nevertheless have reflected the "natural" or more usual metabolic state of the individuals in question. Finally, those factors measured and found to exert significant influence by multiple regression (the "food-interval", and height) are held constant by the analysis to enable the independent and relatively unbiased contribution of the other factors to be defined.

Many of the physiological variables found to have significant simple correlations with the serum-insulin values were shown by multiple regression analysis to have independent associations. The "one hour" blood-sugar level was the major "determinant" of $\log_{10}$ serum-insulin. A non-linear relationship in the crude data (Figure 7) was difficult to interpret since age and percentage desirable weight were interfering variables. The independent association of $\log_{10}$ serum-insulin and blood-sugar, as defined by the multiple regression equation, was more markedly curved, and was almost identical in males and females (Figure 15). Within the "normal" range of blood-sugar levels, a positive linear correlation with $\log_{10}$ serum-insulin was maintained. As with the healthy volunteers (Chapter 3), such a relationship implies "insulin resistance" and indicates that the latter is an important source of variability of serum-insulin levels, independent of the influence of other factors. An important feature to emphasize is that the maximum mean serum-insulin levels, in both sexes, occurred with "one hour" blood-sugar levels of 180 mg/100 ml. Thereafter, with increasing blood-sugar levels, a progressive decline of insulin values was evident. It must be concluded that there is a limit to which "insulin resistance" alone contributes to hyperglycaemia. Beyond this threshold level of blood-sugar, other factors operate so that further hyperglycaemia

is associated with impaired pancreatic secretion of insulin. Blood-sugar levels exceeding 180 mg/100 ml, "one hour" after 50 g. oral glucose) therefore indicate insulin deficiency as a general rule in this population. Thus the levels of blood-sugar chosen by experienced physicians to discriminate abnormal glucose tolerance conform approximately to the levels at which relative insulin deficiency is apparent - for example, diagnostic levels of 180 mg/100 ml at one hour after 50 g. oral glucose (College of General Practitioners 1962); 170 mg/100 ml one hour after a 100 g. oral glucose load (Joslin 1959); and 160 mg/100 ml for a casual post-prandial blood sample (Malins 1968). Further considerations of the concepts of "insulin resistance", and of impaired pancreatic secretion, are discussed later.

Of the various measures of obesity, percentage desirable weight correlated most strongly with $\log_{10}$ serum-insulin, and this was confirmed on multiple regression analysis by the highly significant independent association of weight (with height and other factors held constant). The influence of (relative) weight was more marked in males than females, but in both sexes, the relationship between weight and $\log_{10}$ serum-insulin was found to be linear over the range of values encountered. Thus, as a general rule in the natural population, excessive body weight does not exceed the secretory reserve of the pancreas: no significant curvilinear tendency for serum-insulin to "plateau" with extreme obesity could be discerned. The "insulin resistance" of obesity has been discussed (Chapter 5).

The rise of the "one hour" serum-insulin with age in both sexes (Figure 5) is highly significant, and resolves previous uncertainty about this issue (Crockford et alii 1966; Chlouverakis et alii 1967; Boyns et alii 1969).

The extent of the rise is moderate, and is confirmed as an independent effect of age at 50 years and over. Since there is an accompanying rise of blood-sugar levels with age it appears that insulin resistance (or insensitivity) is a phenomenon of ageing. The recognized deterioration of carbohydrate tolerance in the aged of Western populations therefore does not relate essentially to any impairment of pancreatic secretion or of insulin synthesis, as has been implied (Butterfield 1967) but mainly to impaired action of the circulating insulin. The elevation of insulin levels is "global" rather than involving a segment of the elderly population (Figure 6), thus there is no clear tendency for any of the aged subjects to show pancreatic failure. Although some evidence exists for an accelerated effect of age in the obese and hyperglycaemic, the more subtle interactions of such variables have not been identified further. It is subsequently demonstrated that active subjects, and lean subjects in "ideal" health, do not exhibit rising insulin levels until aged 60 years or more.

Females have higher insulin levels than males. These small but highly significant sex differences have recently been confirmed (Boyns et alii 1969). The more marked elevation of insulin values in younger females probably reflect the influence of female sex hormones (including pregnancy hormones, and possibly the contraceptive pill) but the persistence of significant elevations of insulin in the post-menopausal age ranges suggest that other factors also operate. A separate analysis (unpublished) has suggested that whereas the sex difference is independent of height, it is related to relative weight and fatfold. Thus it is possible that the greater adiposity of females (in the samples analyzed) accounted for their elevated serum-insulin levels, but further work is required.

Serum uric acid showed independent correlations with $\log_{10}$ serum-insulin in both sexes: the cause for this is obscure. Serum uric acid relates to lipid metabolites, particularly triglyceride levels, but also serum cholesterol (Bercowitz 1964). Uric acid levels are elevated in vascular disease (Hall 1964; Breckenridge 1966). Either of these possibilities could account for the observed association of uric acid and insulin, but the nature of the inter-relationships is unexplained. Any substantial alloxan-like effect of uric acid on pancreatic beta cells (Conn et alii 1949) would seem unlikely.

The negative association of serum potassium with the insulin levels one hour after glucose probably reflects one of the recognized effects of insulin action although it is possible that potassium depletion, which is known to cause glucose intolerance (Sagild et alii 1961) does so by impairing insulin's membrane action.

All of the above variables included in the multiple regression equations as determinants of serum-insulin have shown strikingly similar effects in the two sexes. Of the remaining factors, serum urea in males, and serum bicarbonate in females, show significant negative associations. These relationships may reflect an influence of renal function but do not afford an easy interpretation.

THE PROBLEM OF "INSULIN RESISTANCE".

This phenomenon has been suggested repeatedly when high levels of serum-insulin have been found coexisting with normal or elevated levels of blood-sugar. The term "insulin resistance" has been validated since Martin et alii (1968) have shown that in normoglycaemic subjects the height of the serum-insulin level is directly related to insulin insensitivity as measured by insulin tolerance tests. In the present study,

the major positive relationship between serum-insulin and blood-sugar (in the normoglycaemic range) demonstrates this entity, as do the independent contributions of obesity, of age, and to a lesser extent of hyperuricaemia. Vascular disease also is characterized by elevated serum-insulin levels. In searching for a unitary hypothesis to account for this common occurrence of "insulin resistance", consideration must be given to the following:-

Impaired capillary permeability to insulin. The passage of insulin from blood to lymph is delayed as compared to smaller molecules (Rasio et alii 1967), and equilibration takes 10-15 minutes. In diabetics, the sensitivity to injected insulin is proportional to the clinical severity of their vascular disease (Martin and Stocks 1968). The forearm studies of Butterfield et alii (1965) give very suggestive evidence that the impaired action of insulin in diabetics or in subjects with simple obesity can be attributed largely to the failure of insulin to escape from the vascular compartment. No studies of the aged have been reported, but it seems that in these too a similar mechanism could operate. There are various hints, and also provocative reports (Siperstein et alii 1968) that morphological changes including basement membrane thickening can be defined in constitutionally prediabetic subjects, but these have not been linked with any studies of the function of small blood vessels.

The remaining possibilities have less support. Antagonists of insulin action could account for insulin resistance. The synalbumin antagonist is "positive" in diabetes and in vascular disease (Vallance-Owen and Ashton 1963) but there are no reports available in simple obesity or in the aged, and indeed there is doubt concerning the exact identity of this factor. Inactive forms of insulin could

contribute to immunologically measurable insulin, but this does not seem to be an important possibility; for immuno-reactive and biologically active insulin correlate well (Sonksen et alii 1967); and biological assays of insulin-like activity confirm the elevated levels of insulin in obesity. (Samaan et alii 1965) in ageing (Streeten et alii 1965), and in vascular disease (see Chapter 4). Evidence for protein binding of insulin is reviewed on p. such binding appears to be weak and reversible, and this has not been directly implicated in "insulin resistance".

Finally, an insensitivity of target tissues to insulin action could account for failure of circulating insulin to act. The major target tissues for insulin action are muscle and adipose tissue, and the liver. Insensitivity of muscle and fat to insulin action could contribute to peripheral insulin resistance. Although both tissues, from the rat, show roughly comparable "in vitro" sensitivity in the biological assays, it is generally considered that muscle, by virtue of its greater vascularity "in vivo", is more important in the disposition of a glucose load (Butterfield, 1965). There is a suggestion that human forearm muscle "in vivo" shows distinct individual variation for given levels of circulating insulin (Christensen and Orksov 1968) but this could also be due to varying vascular permeability. The large fat cells of obese humans have been shown to be less responsive to insulin than the smaller cells of lean individuals (Salans et alii 1968). This, however, is not likely to be the sole explanation of insulin resistance in obesity, for it does not account for the impaired filtration of insulin from the vascular compartment of the obese.

Least of all is known about the exact role of the liver in glucose homeostasis. Kinetic studies in man (Franckson et alii 1966) do not afford a clear interpretation. Experiments on the dog show that the liver responds to small increases in portal venous insulin by decreasing glucose production (Madison et alii 1960). This finding has been confirmed and expanded by Steele et alii (1968) who have demonstrated that oral glucose loads exert greater effects in diminishing the glucose release from liver than intravenous loads, presumably by virtue of greater insulin secretion. The extent of glucose production by the liver during the overall disposition of a glucose load, is undetermined. Varying sensitivity of liver cells to insulin could, indeed, account for a "central" insulin resistance, in diabetics and obesity with increased glucose production as the result. There is no good evidence for or against this hypothesis: it may prove to be complementary to "peripheral" insulin resistance.

There is some evidence, in the aged, that insulin insensitivity is related to chromium deficiency.(Levine et alii 1968). This appears to be reversible by chromium supplements. Confirmation of these findings in larger numbers of subjects with suitable controls is clearly desirable.

All of the above possibilities reviewed may prove to contribute, to a greater or lesser extent, to the unexplained phenomenon of insulin resistance. Impaired vascular permeability, as a unitary hypothesis, provides a more convenient field for immediate research than pursuit of some of the remaining theories. There are, at present, some positive indications supporting the concept, and no contradictory evidence has yet emerged. The study of vascular permeability, and hereditary and acquired factors related to it, may yield fruitful information about further mechanisms of common diseases.

THE IMPAIRED SECRETORY RESPONSE OF THE PANCREAS.

The general principle derived from the unique independent relationship between blood-sugar and serum-insulin (Figure 15), is that (1) "borderline" hyperglycaemia, with a blood-sugar level of less than 180 mg/100 ml is related essentially to insulin resistance (the positive, ascending part of the curve); whereas (2) "frank" hyperglycaemia exceeding a level of 180 mg/100 ml is associated with insulin deficiency proportional to the height of the blood-sugar. The lack of insulin at one hour probably reflects both a delay in the secretion of insulin initially demonstrated by Yalow and Berson (1960), as well as an overall lack of total insulin relative to the glycaemic stimulus (Seltzer et alii 1967). A preliminary assessment of hospital diabetics (Appendix 2) indicated that the one hour test would most clearly demonstrate this trend. Factors causing the impaired insulin release in more severe diabetics have not been identified. The obvious explanation of the close association between excessive hyperglycaemia and declining insulin levels is that the degree of insulin deficiency accounts for the severity of the diabetes. This, indeed, may prove to be so; and raises various possibilities - that the access of glucose or other secretogues to the beta cell may be restricted; or that the secretory threshold of the beta cell may be elevated by humoral or anatomical factors; or that a true defect of insulin synthesis or release occurs. However, the existence of a fairly well defined threshold of blood-glucose, above which a progressive impairment of insulin secretion may be predicted, suggests that a systematic phenomenon operates. It is possible that the hyperglycaemia per se contributes in some way to delayed insulin release, and this unorthodox viewpoint was pursued.

One means by which high levels of glucose could impair the secretory response of the beta cell is conceivably by the physicochemical effect of hyperosmolality. There are clinical reasons for suspecting that hyperosmolar states are associated with a suppression of the secretion of insulin although insulin is available in the beta cells. In the condition of hyperosmolar coma in diabetes, it has been found that less insulin is required than usually used to treat diabetic ketoacidosis (Danowski and Nabarro 1965) and in some cases very small amounts or no insulin at all has been necessary (Kumar 1968; Hayes and Woods 1968). Those patients recovering from this syndrome are generally found to be quite mild diabetics and thus are presumed to have the ability to produce endogenous insulin (Jackson Forman 1966). Infants with severe dehydration from gastroenteritis frequently show marked hyperglycaemia that resolves with fluid and electrolyte replacement alone (Burman and Claspole 1964; Levin and Geller-Bernstein 1964; Roberts 1964). Other metabolic factors, for example catecholamines, may well be involved in these hyperosmolar states. Further indirect evidence that hyperosmolality may contribute to impairment of insulin release is supported by animal experiments in which rats made hypernatraemic develop glucose intolerance (Nitzan and Zelmanovsky 1968). Excessive osmolality would not appear to inhibit insulin's effect on target tissues, for this has been shown to augment measurements of insulin-like activity on "in vitro" adipose and muscle tissue (Kuzuya et alii 1965).

The moderate elevations of plasma osmolality in the females of the population study were associated with declining insulin levels. This relationship, shown to be independent and highly significant, could provide a clue to the mechanism of hyperglycaemia in hyperosmolar states. While due caution is appropriate in assuming that the relationship implies causality, it is nevertheless entirely feasible that hyperosmolality influences the responsiveness of the beta cell, which has been shown to be very sensitive to extracellular cation concentrations (Milner and Hales 1968). In studies reported by Cerasi and Luft (1967) some normal subjects with a defective insulin response to sustained intravenous hyperglycaemia do not show any substantial insulin secretion until the blood-sugar level falls below 200-250 mg/100 ml. The possibility exists, therefore, and deserves further study, that the hyperosmolality of hyperglycaemia contributes directly to the delayed insulin secretion characteristic of maturity-onset diabetes.

The multiple regression equations (Table 18) accounted for 34% of the variability of the serum-insulin levels in males, and 28% in females. (Corresponding values for the "unbiased" index of determination for other physiological variables similarly analyzed have been 10-20% for blood-sugar and serum cholesterol levels, and 20-30% for casual blood-pressure readings.) Thus the findings provide considerable support for the biological validity of serum-insulin levels as measured by immunological assay, especially in view of the comparatively uncontrolled conditions of the experiment. It must also be taken into consideration that a number of important factors known to influence glucose tolerance and insulin levels, such as growth hormone, cortisol, and catecholamine secretion were not measured. One third, therefore, of the wide range of

insulin values could be "explained", and a substantial part of this variability appears to be related to "insulin resistance", a phenomenon that perhaps deserves more attention in the interpretation of the levels of circulating insulin.

TABLE 10. Distribution of the absolute insulin values ($\mu\text{U}/\text{ml.}$) "one hour" after 50g. glucose in 1629 males and 1684 females, showing significant departures from the expected normal distribution.

<u>"ONE HOUR" INSULIN LEVELS IN 3313 SUBJECTS.</u>			
<u>RANGE</u>		<u>NO.of MALES</u>	<u>NO.of FEMALES</u>
Less than 10 $\mu\text{U}/\text{ml.}$:		20	9
10 - 29	" :	455	317
30 - 49	" :	515	559
50 - 69	" :	317	373
70 - 89	" :	141	191
90 - 109	" :	72	94
110-129	" :	43	63
130-149	" :	23	34
150-169	" :	11	11
170-189	" :	12	11
190-209	" :	3	4
210-309	" :	9	12
310-400	" :	8	6
TOTAL:		1629	1684
Mean $\pm$ S.D.	:	54 $\pm$ 41 $\mu\text{U}/\text{ml.}$	60 $\pm$ 41 $\mu\text{U}/\text{ml.}$
Median	:	41 $\mu\text{U}/\text{ml.}$	48 $\mu\text{U}/\text{ml}$
χ^2 *		>100 ($p < 0.001$)	>100 ($p < 0.001$)

* Chi-squared test for "goodness of fit" with normal distribution (5 degrees of freedom) using number of subjects demarcated by mean $\pm$ 1 standard deviation
 $\pm$ 1 to 2 standard deviations
 $\geq$ 2 standard deviations
 in the same manner as shown in Table 11.

TABLE 11. (A) The means, medians, and standard deviations for the $\log_{10}$ serum-insulin values of 1629 males and 1684 females "one hour" after glucose.

(B) The observed compared with the expected distribution of $\log_{10}$ serum-insulin values as delimited by standard deviations from the mean. The chi-squared test has 5 degrees of freedom.

(A). LOG_{10} SERUM-INSULIN VALUES:

SUBJECTS	MEAN (M)	MEDIAN	STANDARD DEVIATIONS (S.D.)
1629 Males :	1.625 (42 μ U/ml.)	1.686 (41 μ U/ml.)	0.279
1684 Females:	1.686 (49 μ U/ml.)	1.682 (48 μ U/ml.)	0.262

(B). DISTRIBUTION OF LOG_{10} SERUM INSULIN:

NUMBER OF SUBJECTS WITH VALUES IN THE RANGE: -

Less than (M-2SD)	(M-1SD) to (M-2SD)	(M to (M-1SD)	(M to (M+1SD)	(M+1SD) to (M+2SD)	Greater than (M+2SD)	χ^2 (d.f.=5)
<u>1629 Males</u>						3.9 (p=0.5-0.6)
Obs- erved ..30	224	585	559	191	40	
Expec- ted ..37.1	221.4	556.0	556.0	221.4	37.1	
<u>1684 Females</u>						3.1 (p=0.6-0.7)
Obs- erved ..34	213	603	592	206	36	
Expec- ted ..38.4	228.9	574.7	574.7	228.9	38.4	

TABLE 12. Mean values $\pm$ standard error of the "one hour" $\log_{10}$ serum-insulin levels in males and females by 10-year age groups.

MEAN LOG ₁₀ SERUM INSULIN $\pm$ 1 S.E. (N = NO. OF SUBJECTS)					
AGE (years)	<u>MALES</u>		<u>FEMALES</u>		SIGNIFI- CANCE p. by "T" TEST
21 - 29:	1.531 $\pm$ 0.015	N = (255)	1.632 $\pm$ 0.014	N = (271)	p<0.001
30 - 39:	1.560 $\pm$ 0.017	N = (279)	1.622 $\pm$ 0.015	N = (320)	p<0.01
40 - 49:	1.591 $\pm$ 0.015	N = (345)	1.641 $\pm$ 0.015	N = (355)	p<0.05
50 - 59:	1.637 $\pm$ 0.015	N = (313)	1.697 $\pm$ 0.014	N = (320)	p<0.01
60 - 69:	1.728 $\pm$ 0.016	N = (264)	1.781 $\pm$ 0.015	N = (245)	p<0.05
70 :	1.757 $\pm$ 0.022	N = (173)	1.827 $\pm$ 0.019	N = (173)	p<0.05
TOTAL :	1.625 $\pm$ 0.007	(1629)	1.685 $\pm$ 0.006	(1684)	p<0.001

TABLE 13. Mean values for the "one hour" blood-sugar levels (mg/100ml.), percentage desirable weight, and mid-triceps fat-fold (mm.) in males and females by 10 year age groups.

AGE (years)	"ONE HOUR" BLOOD-SUGAR (mg/100ml.)		PERCENT DESIRABLE WEIGHT		MID-TRICEPS FAT-FOLD (mm.)	
	<u>MALES - FEMALES</u>		<u>MALES - FEMALES</u>		<u>MALES - FEMALES</u>	
21 - 29:	88	86	107	113	9	19
30 - 39:	93	92	109	116	10	21
40 - 49:	97	103	114	120	11	23
50 - 59:	108	108	113	124	10	24
60 - 69:	113	120	116	126	11	23
70 + :	125	134	112	124	11	21

VARIABLE (and units used)

	1629 MALES			1684 FEMALES		
	Mean ± 1 S.D.	(Min.	Max.)	Mean ± 1 S.D.	(Min.	Max.)
Blood sugar (mg/100ml.)	103 ⁺ 37	(42	-	105 ⁺ 39	(25	-
Age (yrs)	48 ⁺ 16	(20	-	48 ⁺ 16	(21	-
% desirable weight	112 ⁺ 15	(62	-	120 ⁺ 21	(73	-
Fat fold (mm.)	10 ⁺ 4	(2	-	22 ⁺ 7	(5	-
Weight (kilos)	73 ⁺ 11	(42	-	64 ⁺ 11	(35.8	-
Height (cm.)	172 ⁺ 7	(131	-	160 ⁺ 6	(134	-
Syst.blood pressure (mm.Hg.)	147 ⁺ 24	(94	-	150 ⁺ 29	(86	-
Diast.blood pressure (mm. Hg.)	79 ⁺ 13	(10	-	81 ⁺ 14	(24	-
Serum uric acid (mg./100ml.)	5.5 ⁺ 1.1	(0.3	-	4.5 ⁺ 1.1	(1.6	-
Serum Cholesterol (mg./100ml.)	251 ⁺ 44	(117	-	262 ⁺ 50	(127	-
S.Sodium (mEq/L)	142 ⁺ 3	(120	-	142 ⁺ 3	(118	-
S.Chloride (mEq/L)	104 ⁺ 3	(82	-	105 ⁺ 3	(81	-
S.Potassium (mEq/L)	4.4 ⁺ 0.5	(30	-	4.2 ⁺ 0.5	(3.0	-
S.Bicarbonate (mEq/L)	25 ⁺ 2	(11	-	25 ⁺ 2	(14	-
S.Urea (mg/100ml.)	34 ⁺ 9	(15	-	29 ⁺ 8	(6	-
S.Calcium (mEq/L)	9.9 ⁺ 0.4	(6.8	-	9.9 ⁺ 0.4	(7.6	-
S.Magnesium (mEq/L)	2.1 ⁺ 0.16	(1.5	-	2.1 ⁺ 0.16	(1.5	-
"Food-interval" (minutes)	183 ⁺ 99	(15	-	184 ⁺ 98	(11	-
"Drink-interval" (minutes)	65 ⁺ 10	(15	-	66 ⁺ 9	(20	-
Time of day (hours)	15.0 ⁺ 3.6	(8.6	-	14.7 ⁺ 3.4	(8.5	-

TABLE 14. Physiological variables measured in the Busselton Population Study (1966) including the means standard deviations, and the absolute ranges of values obtained in males and females.

TABLE 15. Physiological variables related to the "one-hour" $\log_{10}$ serum insulin values. The correlation coefficient (r) is shown for each variable, and its significance. (The units of the physiological variables listed are as shown in Table 14.)

<u>CORRELATIONS WITH THE "ONE-HOUR" $\log_{10}$ SERUM-INSULIN</u>				
VARIABLE:	<u>1629 MALES:</u>		<u>1684 FEMALES:</u>	
	r	Significance	r	Significance
Blood-sugar	0.331	(p<0.001)	0.339	(p<0.001)
Age	0.271	(p<0.001)	0.253	(p<0.001)
% desirable weight	0.319	(p<0.001)	0.226	(p<0.001)
Fat-fold	0.263	(p<0.001)	0.173	(p<0.001)
Weight	0.234	(p<0.001)	0.158	(p<0.001)
Height	-0.106	(p<0.001)	-0.154	(p<0.001)
Systolic B.P.	0.184	(p<0.001)	0.219	(p<0.001)
Diastolic B.P.	0.072	(p<0.01)	0.072	(p<0.01)
S. uric acid	0.179	(p<0.001)	0.183	(p<0.001)
S. Cholesterol	0.101	(p<0.001)	0.113	(p<0.001)
S. Sodium	-0.041	(N.S.)	-0.035	(N.S.)
S. Chloride	-0.008	(N.S.)	-0.078	(p<0.01)
S. Potassium	-0.051	(p<0.05)	-0.034	(N.S.)
S. Bicarbonate	-0.047	(N.S.)	-0.055	(p<0.05)
S. Urea	-0.020	(N.S.)	0.109	(p<0.001)
S. Calcium	-0.027	(N.S.)	-0.014	(N.S.)
S. Magnesium	-0.058	(p<0.05)	-0.016	(N.S.)
"Food-interval"	-0.191	(p<0.001)	-0.105	(p<0.001)
"Drink-interval"	-0.113	(p<0.001)	-0.035	(N.S.)
Time of day	-0.058	(p<0.05)	-0.018	(N.S.)

TABLE 16.

Coefficients of correlation between $\log_{10}$ serum-insulin, blood-sugar, percentage desirable weight, and age in 1629 males and 1684 females.

LINEAR CORRELATION COEFFICIENTS (r)				
	$\log_{10}$ Insulin	Blood sugar	% D.W.	AGE
$\log_{10}$ Insulin:		*** .331	*** .319	*** .271
Blood-sugar:	*** .339		*** .098	*** .312
% D.W.:	*** .226	*** .157		.145
Age:	*** .253	*** .379	*** .204	

FEMALES

M
A
L
E
S

p<0.001.

TABLE 17. Coefficients of correlation between $\log_{10}$ serum-insulin, blood sugar, the time of day, and the intervals from glucose load, and from last taking food.

LINEAR CORRELATION COEFFICIENTS (r)					
	Log ₁₀ INSULIN	BLOOD- SUGAR	DRINK INTER- VAL	FOOD INTER- VAL	TIME OF DAY
Log ₁₀ Insulin:		+.331 ^{***}	-.113 ^{***}	-.191 ^{***}	-.058 [*]
Blood-sugar:	+.339 ^{***}		-.078 ^{**}	+.097 ^{**}	+.069 ^{**}
Drink Interval:	-.035	+.025		+.053 [*]	+.074 ^{**}
Food Interval:	-.105 ^{***}	+.113 ^{***}	+.106 ^{***}		+.053 [*]
Time of Day:	-.018	+.119 ^{***}	+.123 ^{***}	+.026	

M
A
L
E
S

FEMALES

* p<0.05
 ** p<0.01
 *** p<0.001

TABLE 18.

Results of multiple regression analysis of factors determining $\log_{10}$ serum-insulin in males and females. The form of the equation is described in the Appendix. The units for the stated factors are as shown in Table 14. The Student's "T" value indicates the significance of the regression coefficient for the orthogonalized variables.

MULTIPLE REGRESSION ANALYSIS OF THE "ONE HOUR" $\log_{10}$ SERUM-INSULIN

1629 MALES

1684 FEMALES

FACTOR	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE
Blood-Sugar	+0.957 x 10^{-2}	14.9 (p<0.001)	+0.110 x 10^{-1}	16.8 (p<0.001)
(Blood-Sugar) ²	-0.372 x 10^{-4}	6.3 (p<0.001)	-0.441 x 10^{-4}	7.6 (p<0.001)
(Blood-Sugar) ³	+0.415 x 10^{-7}	3.1 (p<0.01)	+0.502 x 10^{-7}	4.2 (p<0.001)
Age	-0.145 x 10^{-2}	7.3 (p<0.001)	-0.706 x 10^{-2}	3.4 (p<0.001)
(Age) ²	+0.728 x 10^{-4}	3.5 (p<0.001)	+0.891 x 10^{-4}	4.6 (p<0.001)
Food-interval	+0.141 x 10^{-3}	12.4 (p<0.001)	-0.138 x 10^{-2}	10.5 (p<0.001)
(Food-interval) ²	-0.693 x 10^{-5}		+0.167 x 10^{-5}	5.9 (p<0.001)
(Food-interval) ³	+0.126 x 10^{-7}	6.4 (p<0.001)		
Drink-interval	-0.174 x 10^{-2}	3.0 (p<0.01)		
Height	-0.620 x 10^{-3}	6.5 (p<0.001)	-0.420 x 10^{-3}	4.4 (p<0.001)
Weight	+0.736 x 10^{-3}	12.1 (p<0.001)	+0.393 x 10^{-3}	7.6 (p<0.001)
S.Uric Acid	+0.209 x 10^{-1}	3.9 (p<0.001)	+0.169 x 10^{-1}	3.2 (p<0.01)
S.Urea	-0.246 x 10^{-2}	4.0 (p<0.001)		
S.Bicarbonate			-0.994 x 10^{-2}	4.2 (p<0.001)
S.Potassium	-0.470 x 10^{-1}	4.4 (p<0.001)	-0.280 x 10^{-1}	2.5 (p<0.05)
Y-INTERCEPT	+1.961		+1.998	

MULTIPLE CORRELATION COEFFICIENT (R)

0.589

0.535

PROPORTION OF VARIABILITY

ASCRIBABLE TO REGRESSION (A)

0.342

0.281

MEAN $\log_{10}$ SERUM-INSULIN

1.625

1.686

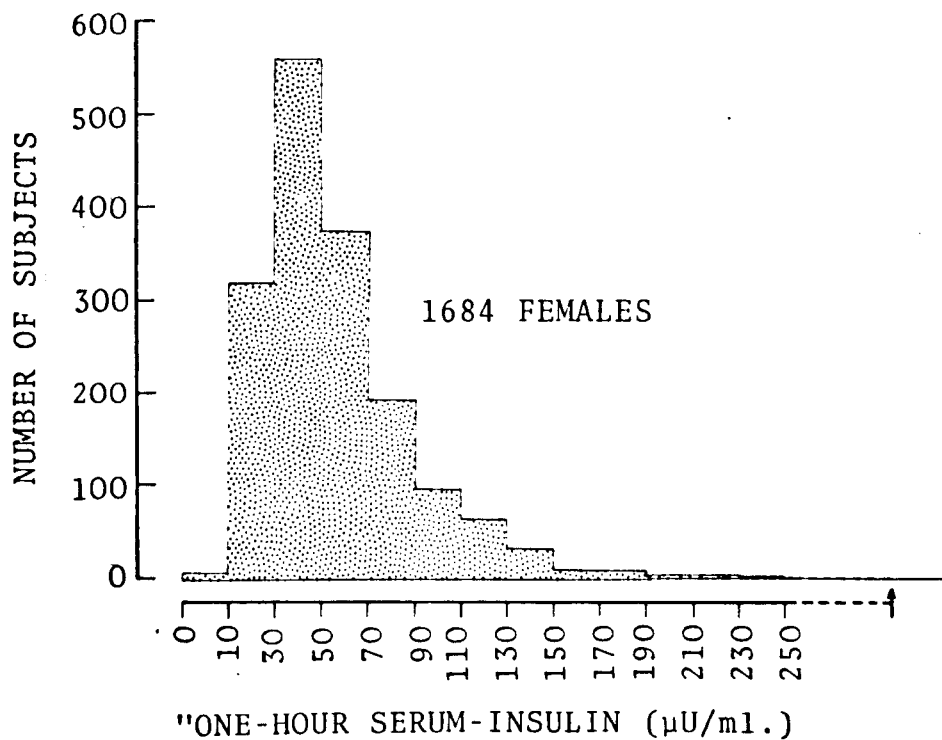
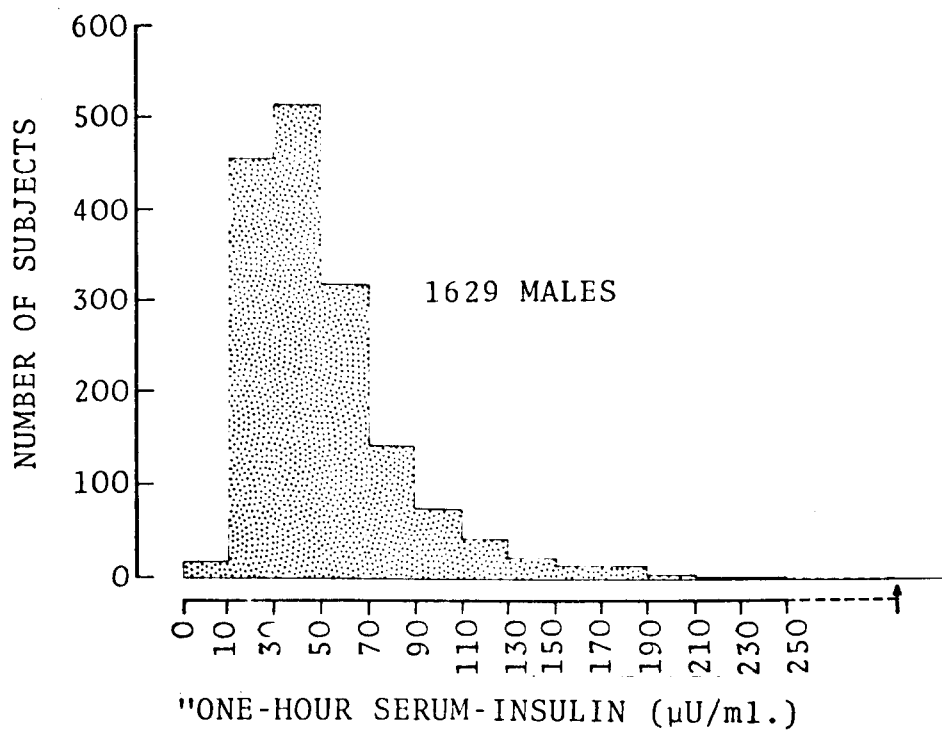


FIGURE 2.

Distribution of the absolute insulin values "one hour" after 50g. glucose in 3313 subjects is unimodal and positively skewed.

PERCENTILES

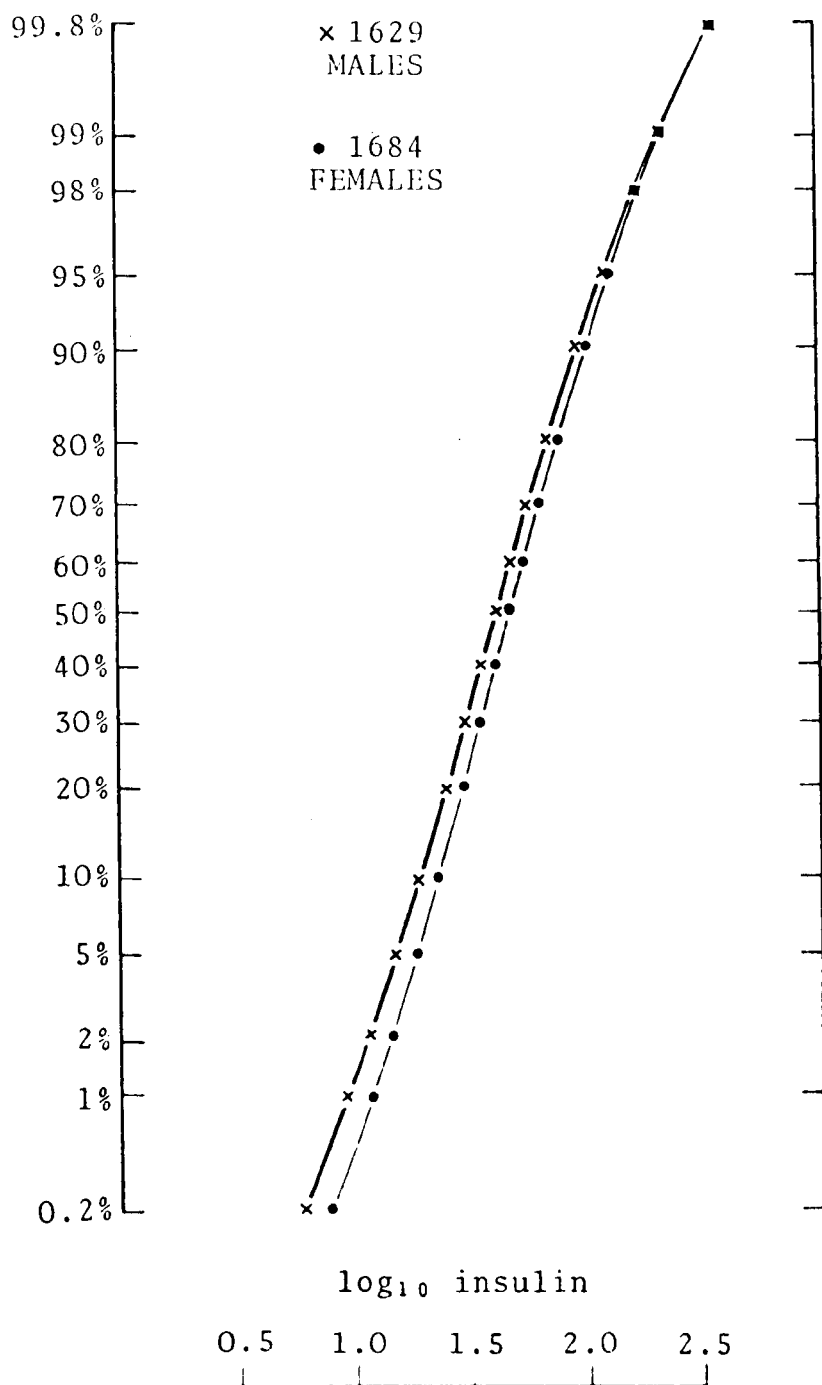


FIGURE 3.

Percentile distribution of the log₁₀ insulin values for 1629 males and 1684 females, plotted on a linear probability scale.

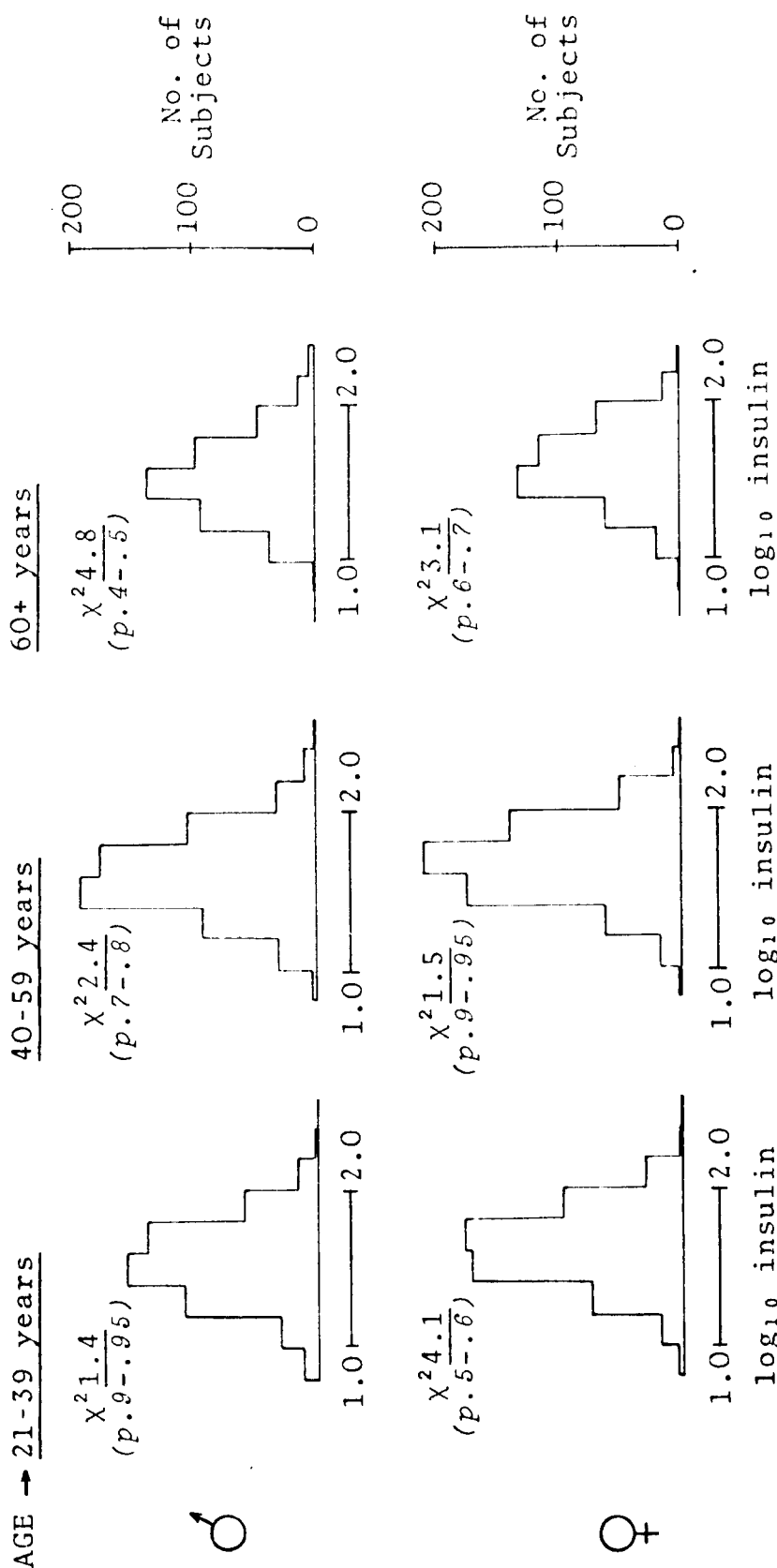


FIGURE 4. Distribution of "one-hour" log₁₀ serum-insulin values in males and females by age groups. Results of the chi-squared test (5 degrees of freedom) indicate closeness of fit with the expected normal distribution as performed in Table 11.

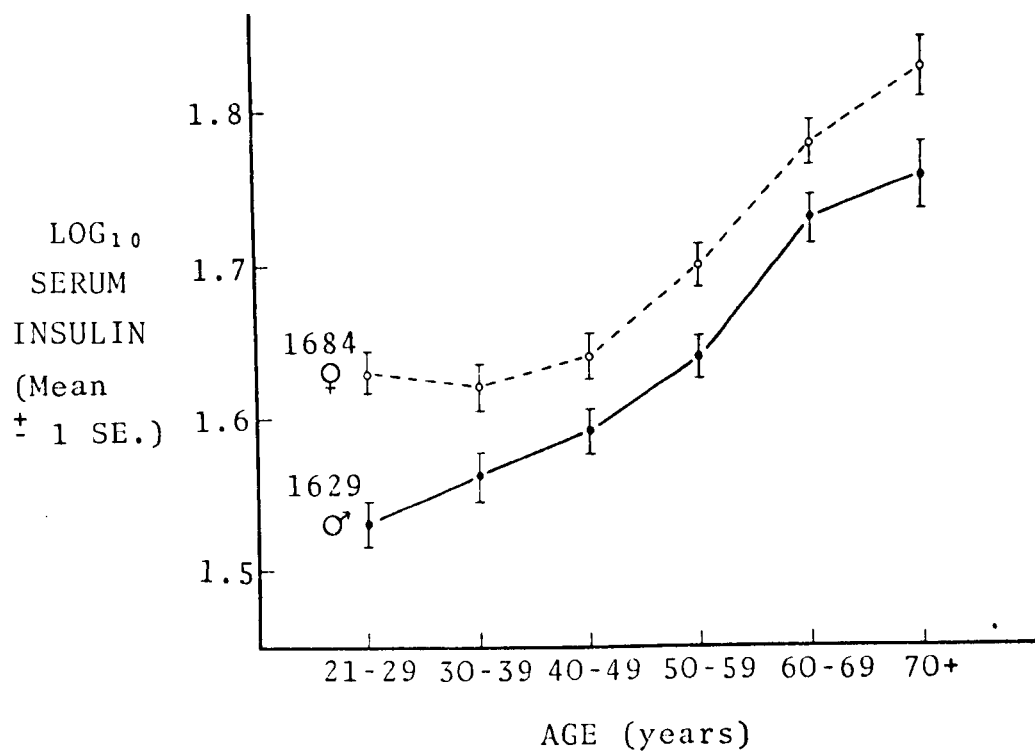


FIGURE 5. Mean "one-hour" $\log_{10}$ serum-insulin ($\pm$ 1 standard error) of males and females in 10 year age groups.

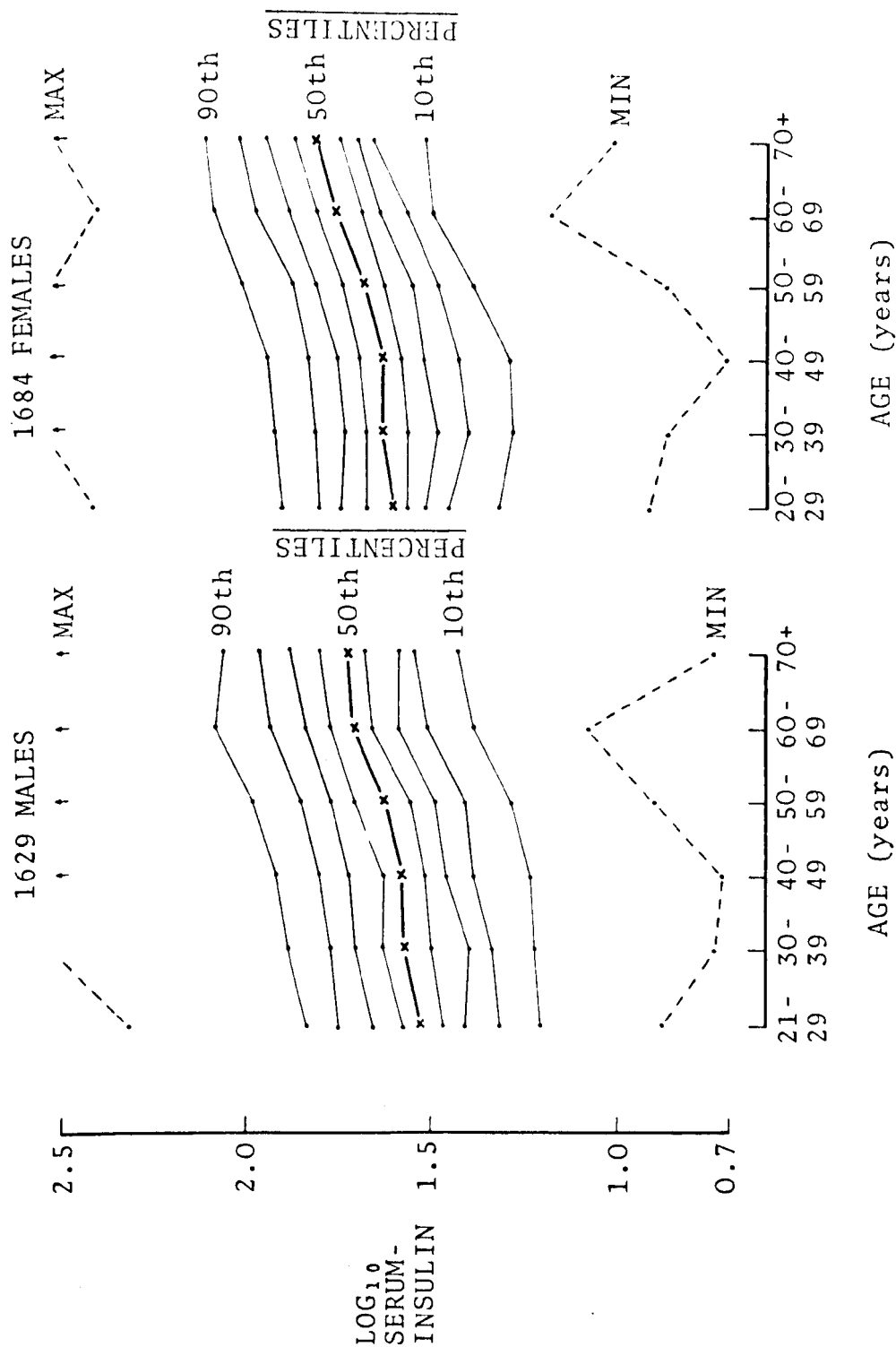
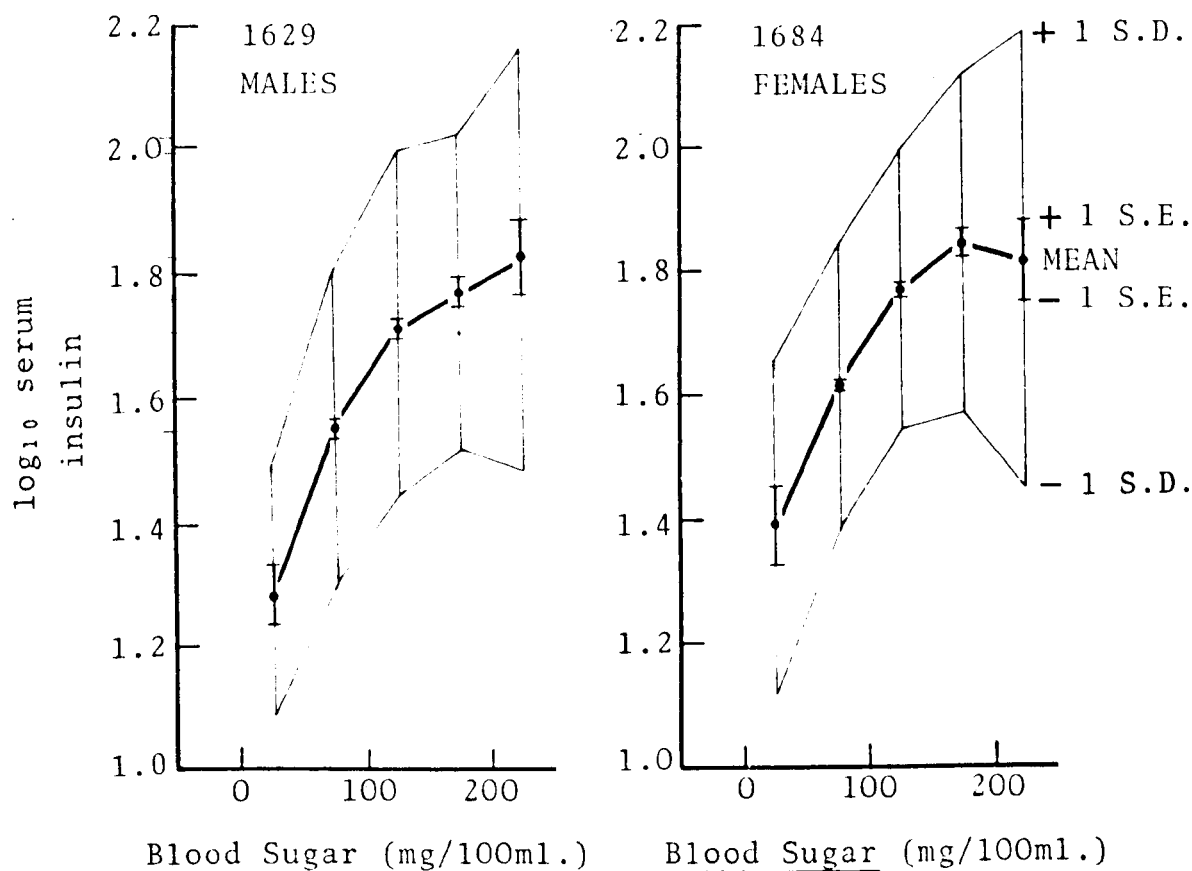


FIGURE 6. Percentiles of the "one-hour" $\log_{10}$ serum-insulin values in 10 year age groups of males and females. All ranges show a parallel trend.



Total No:	16	94	053	99	35
Mean % DW:	10.4	11.1	11.3	11.2	11.9
Mean Age:	43	44	52	59	62

17	93	954	215	432
119	118	122	127	127
37	43	52	59	66

FIGURE 7.

Mean "one-hour" serum-insulin values according to (50mg/100ml) classes of blood-sugar, with the range of 1 standard error (SE) and 1 standard deviation (SD). The mean percent desirable weight and mean age of subjects are tabulated.

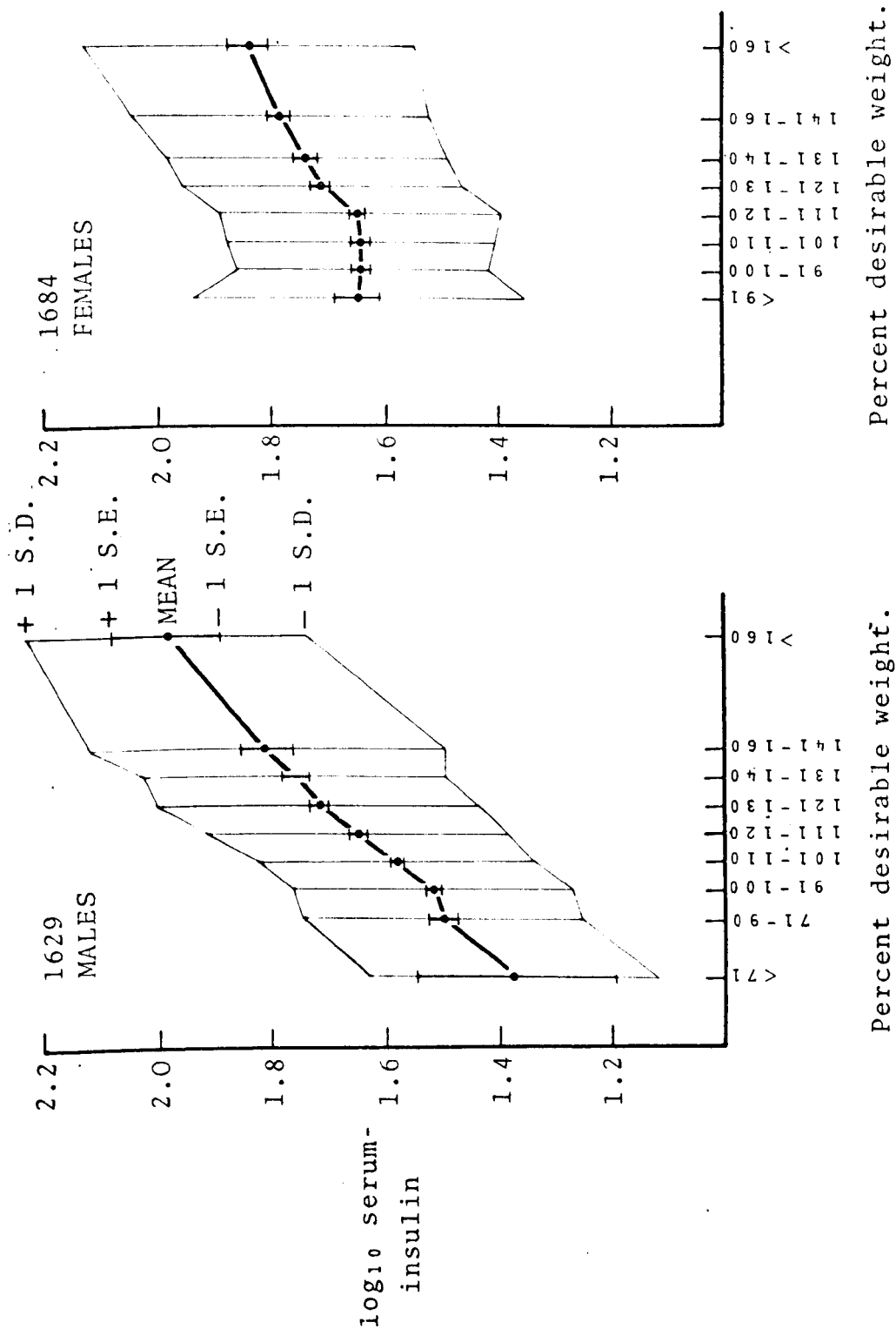


FIGURE 8.

Mean "one hour" serum-insulin values according to classes of percentage desirable weight, with the range of 1 standard error (SE) and 1 standard deviation (SD).

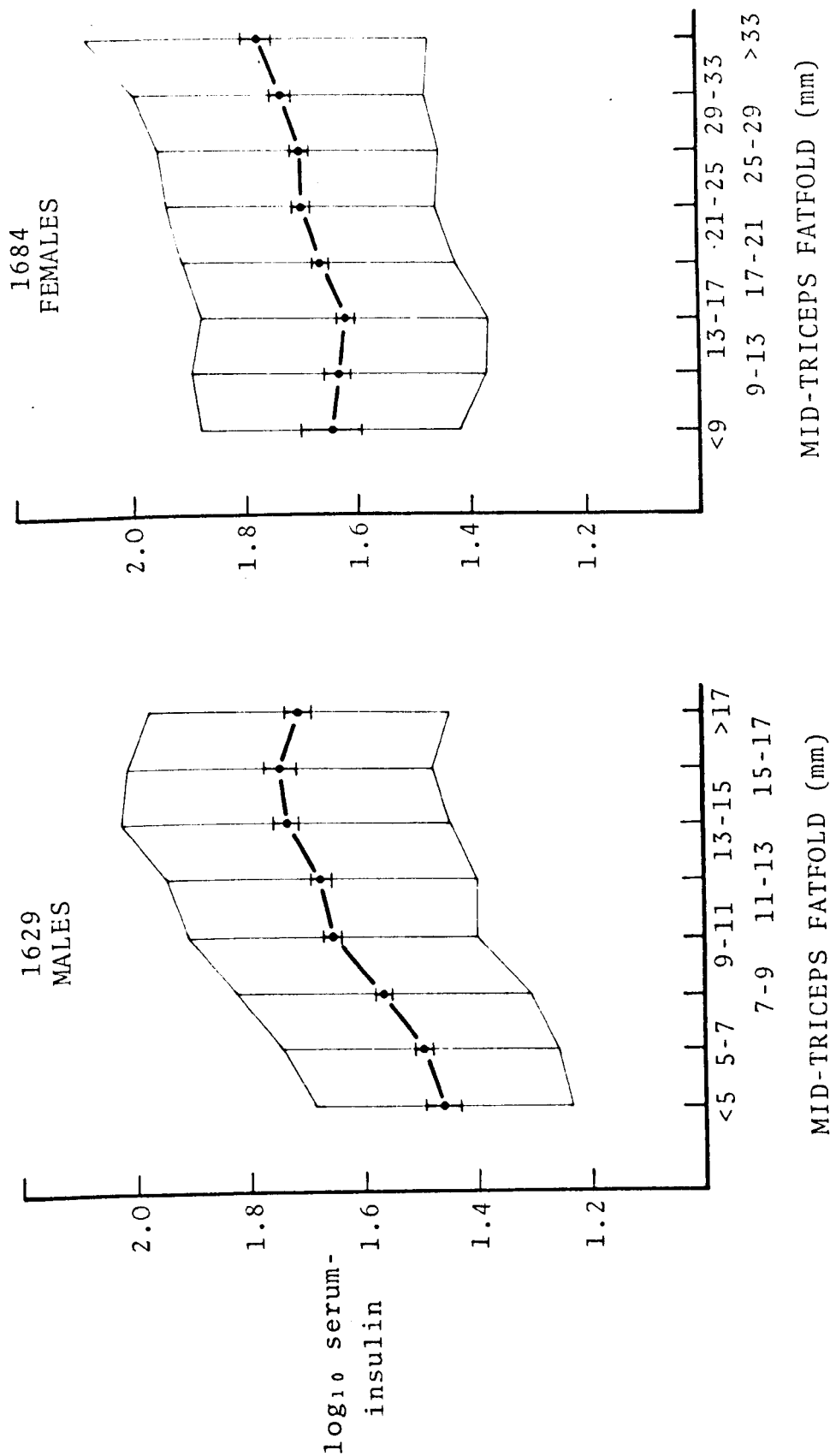


FIGURE 9.

Mean "one hour" serum-insulin values according to classes of mid-triceps fat-fold, with the range of one standard error and one standard deviation.

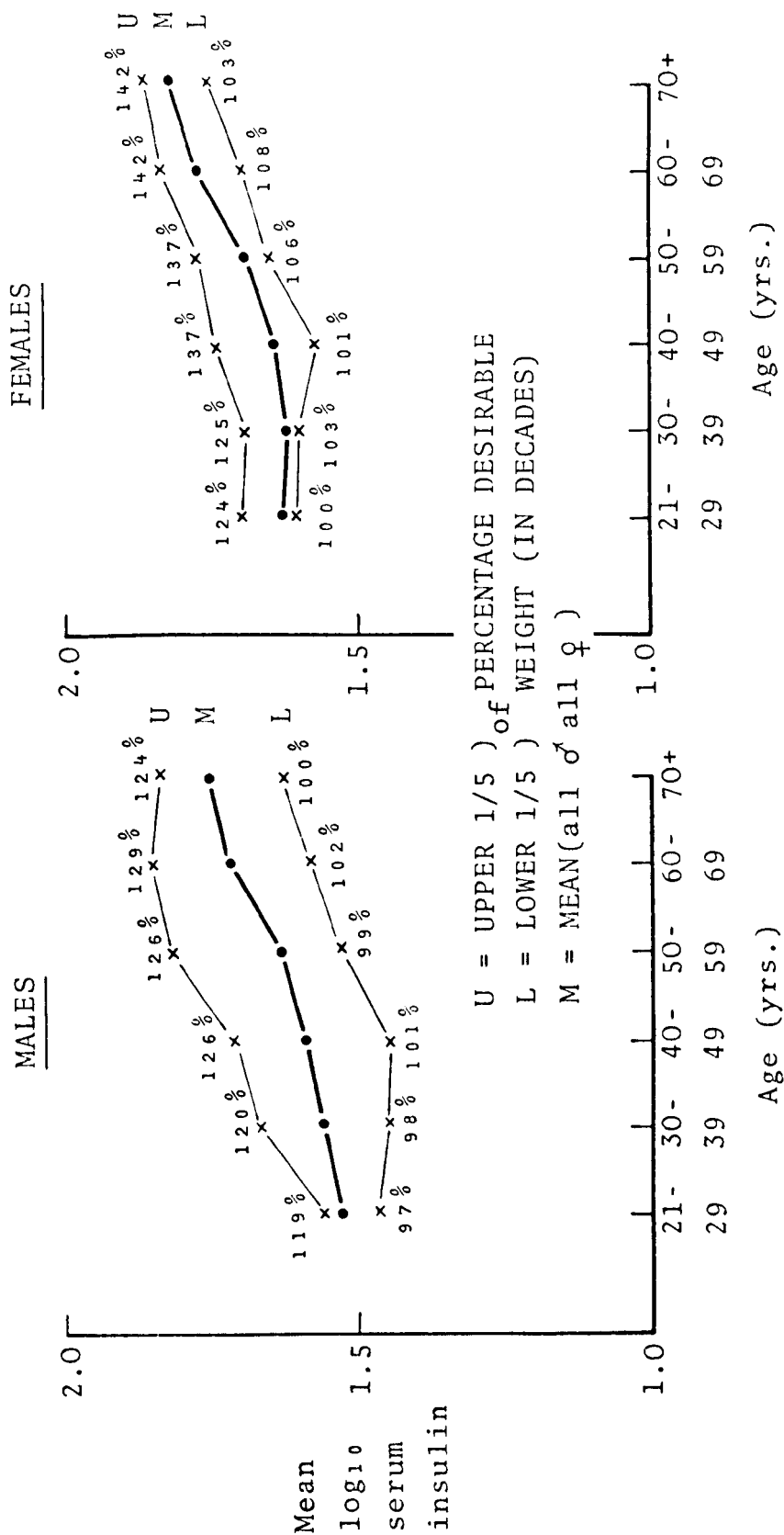


FIGURE 10. Mean "one hour" serum-insulin values for subjects with upper and lower quintiles of percentage desirable weight in 10-year age groups, compared with the overall mean. The percentage figures indicate the 20th and 80th percentile values.

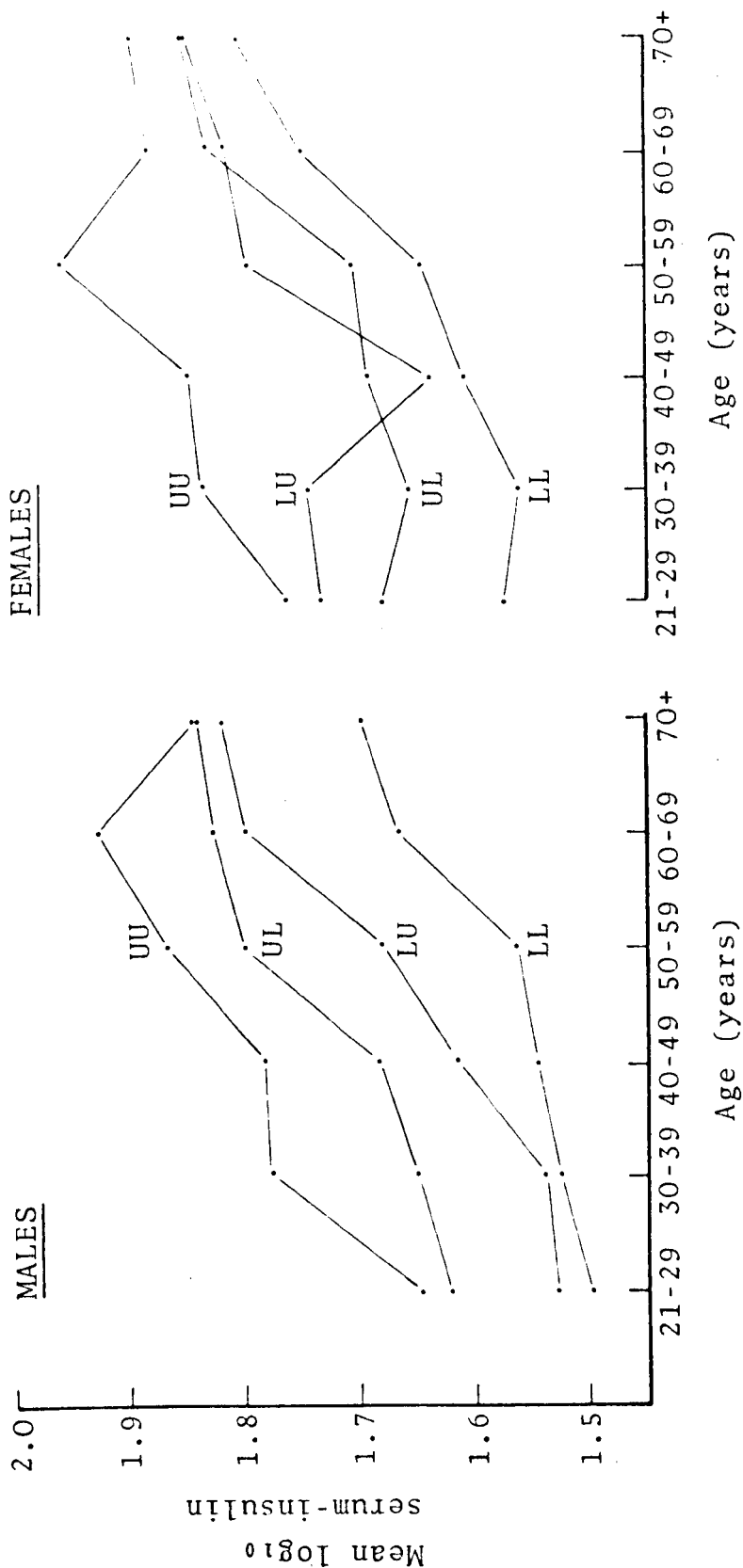


FIGURE 11. Mean "one-hour" serum-insulin values for subjects with the following ranges of percentage desirable weight (%DW) and blood-sugar levels (BSL): -

UU = "upper range" %DW + "upper range" BSL (5% of population)
 UL = "upper range" %DW + "lower range" BSL (16%)
 LU = "lower range" %DW + "upper range" BSL (16%)
 LL = "lower range" %DW + "lower range" BSL (63%)

The sex and decade specific 80th percentile values divide "upper" from "lower" ranges.

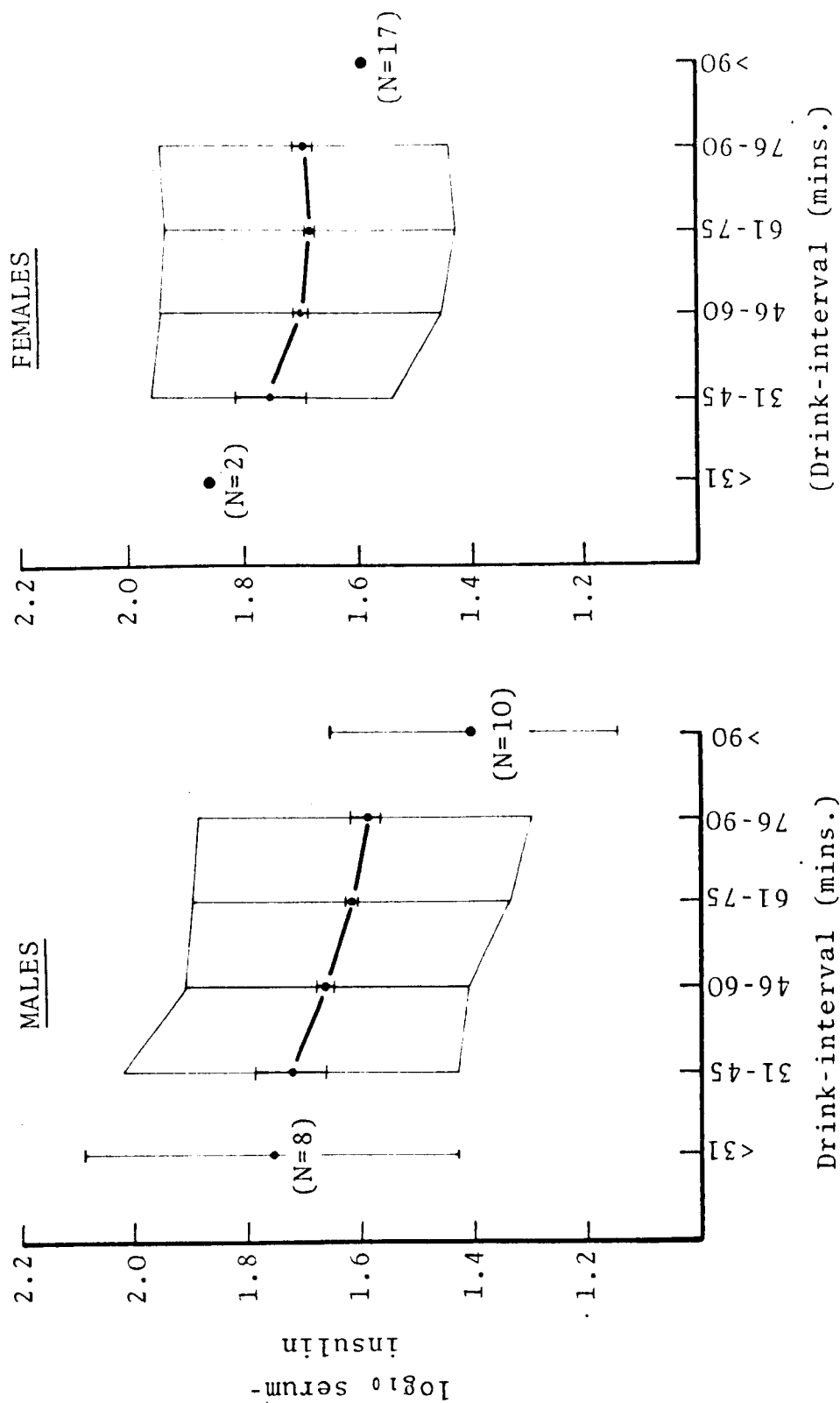


FIGURE 12.

Mean serum-insulin values according to the interval from glucose load to venepuncture (the "drink-interval"), with the range of 1 standard error and 1 standard deviation.

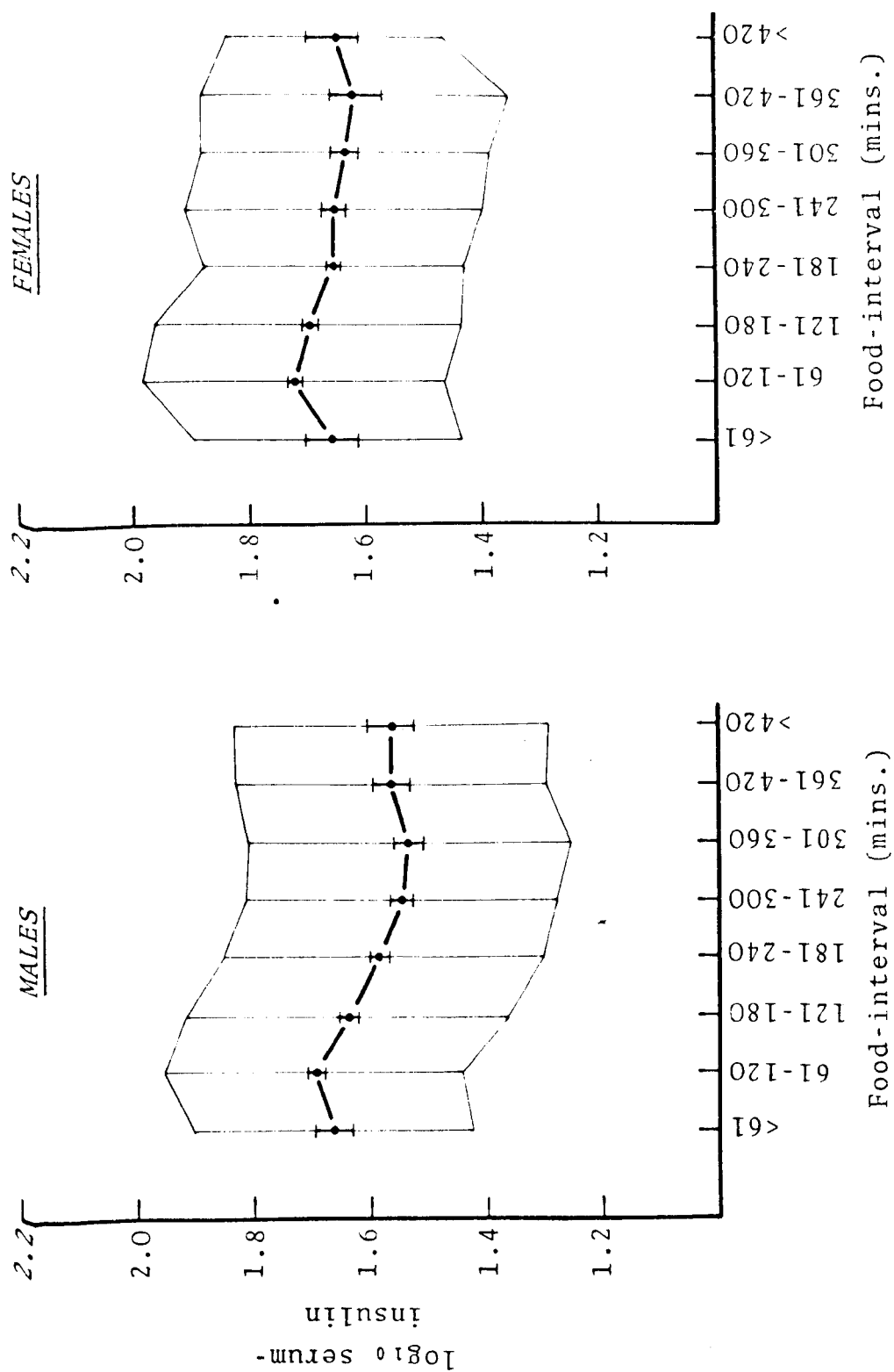


FIGURE 13. Mean serum-insulin values of subjects according to the interval from last taking food or calorie containing fluids (the "food-interval"), with the range of 1 standard error and 1 standard deviation.

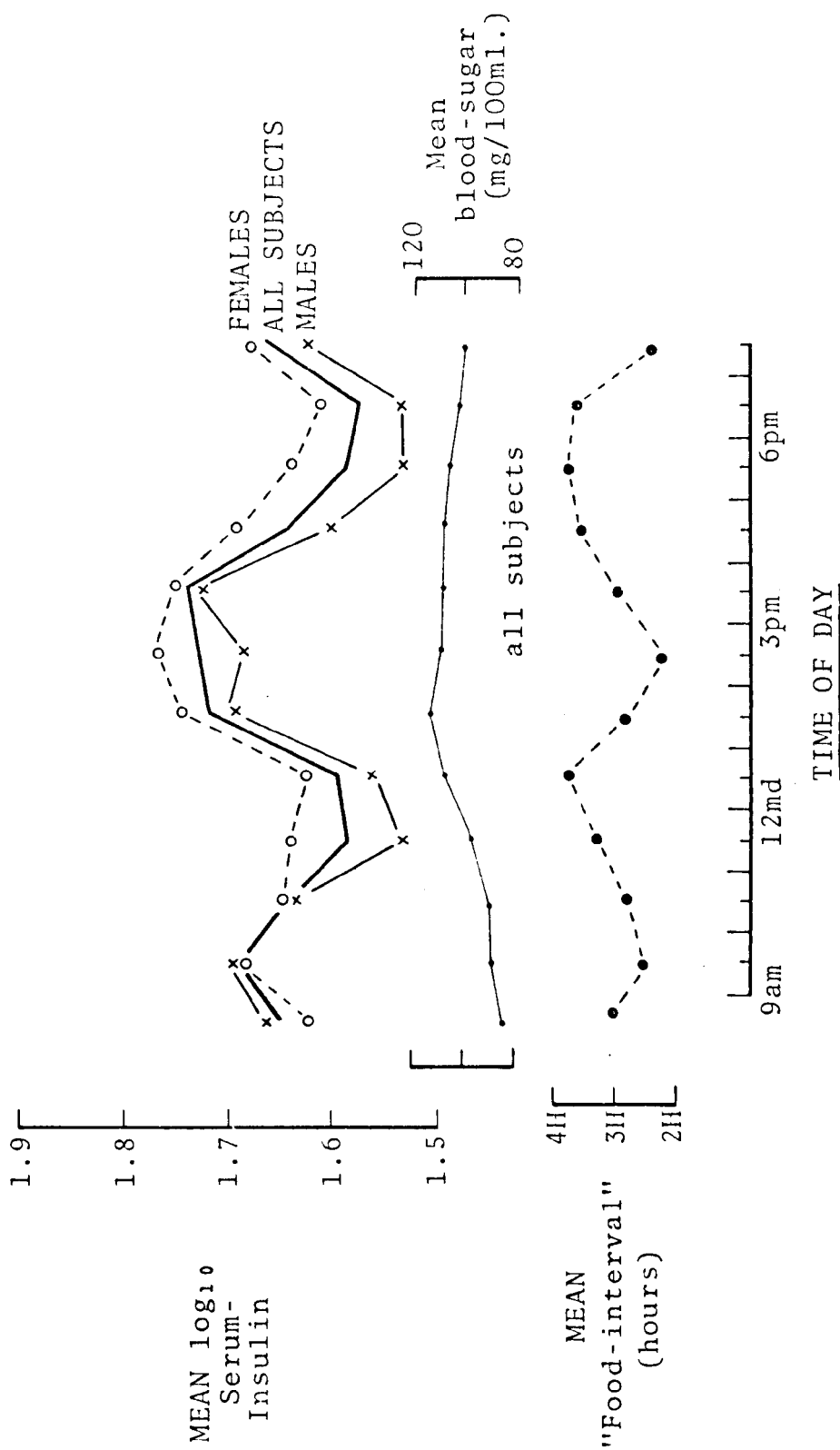


FIGURE 14. Effect of time of day on mean insulin values and mean blood-sugar levels ("one hour" after glucose), and the association observed with the mean "food interval". The variation of serum-insulin has a close inverse relationship with the "food-interval".

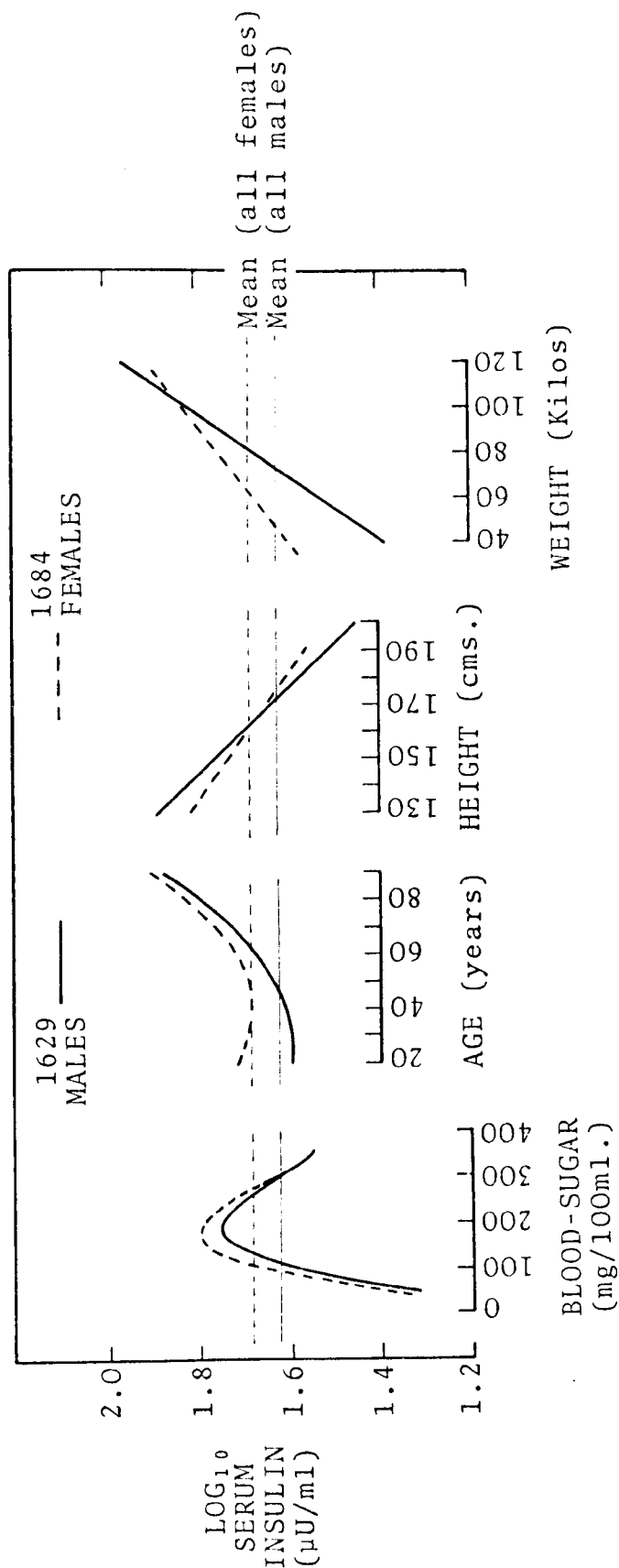


FIGURE 15. The independent associations of blood-sugar, age, height, and weight, with the "one hour" $\log_{10}$ serum-insulin values in males and females, as obtained from the multiple regression analysis in Table 18.

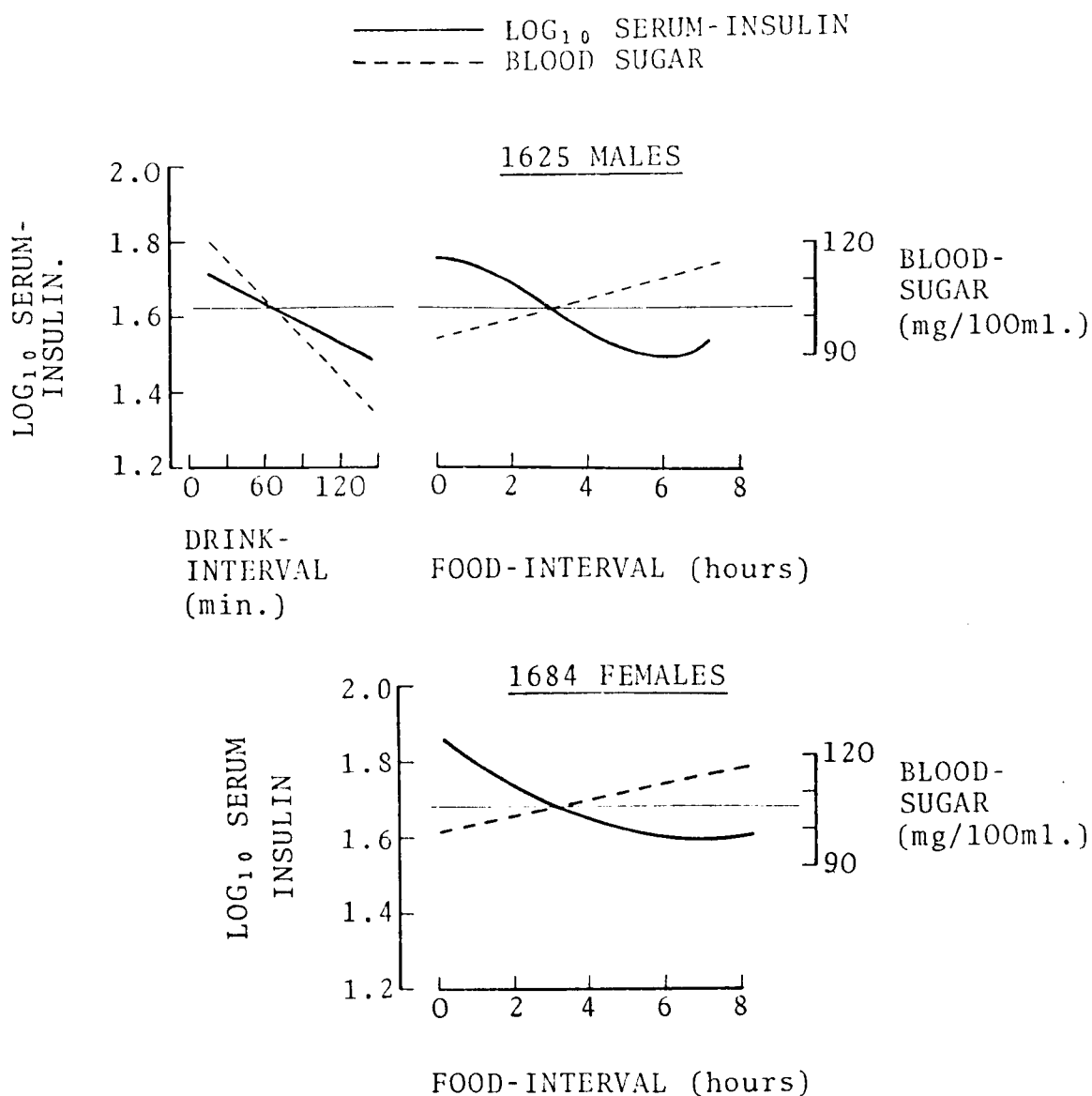


FIGURE 16.

The independent effects of the interval from glucose load and the interval from last taking food on the log₁₀ serum-insulin values of males and females, as obtained from the multiple regression analyses in Table 18

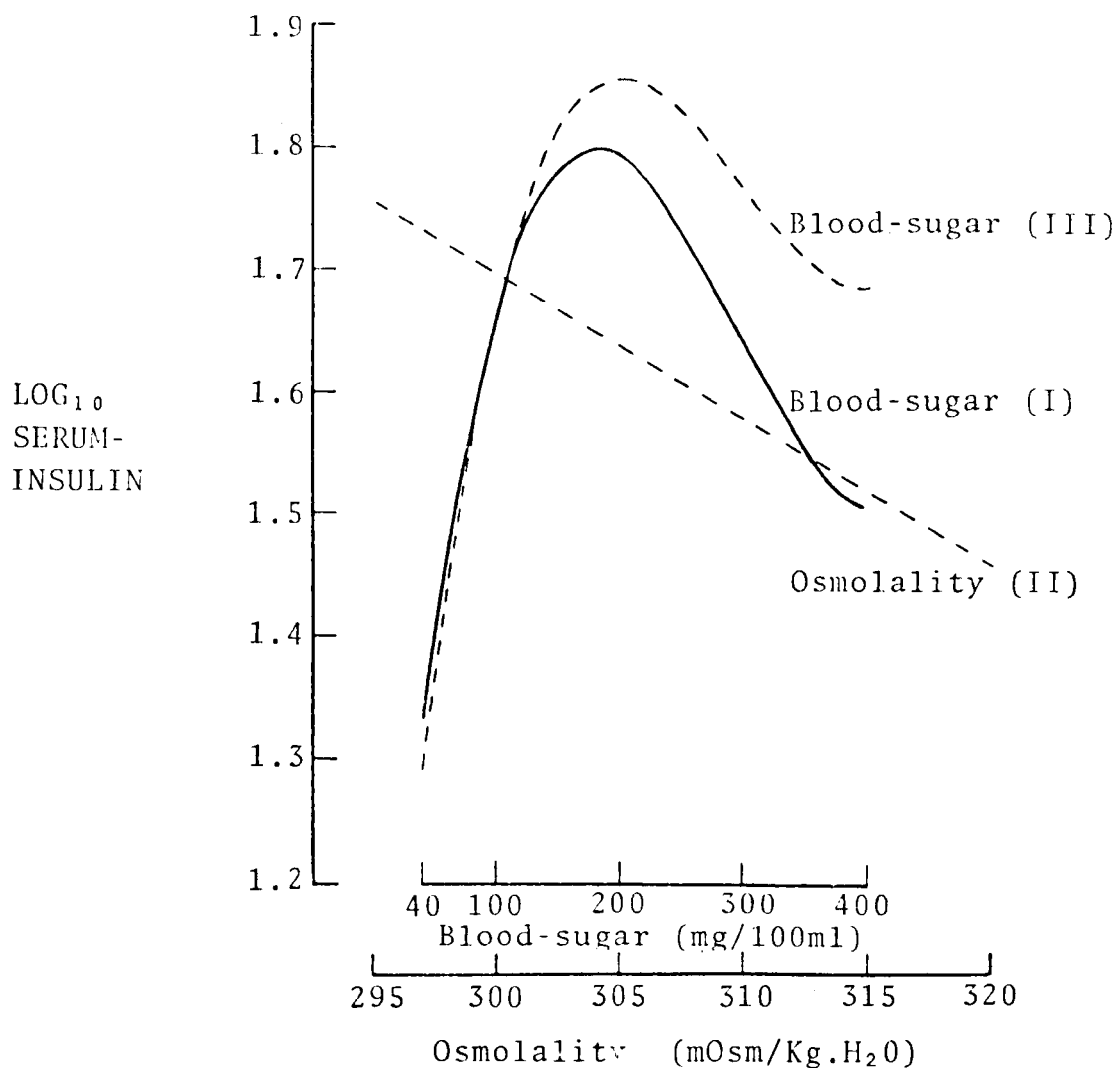


FIGURE 17.

The effect of plasma osmolality on the "one hour" serum-insulin in females, as determined by multiple regression analysis. The initial regression equation (Table 18) defined the independent effect of the blood-sugar levels with $\log_{10}$ serum-insulin (curve I). When plasma osmolality was included as a possible determining variable, it had significant negative association on $\log_{10}$ serum-insulin (line II) and the relationship between blood-sugar and $\log_{10}$ serum-insulin (curve III) was altered.

RESULTS

II. THE "ONE-HOUR" SERUM-INSULIN IN HEALTH AND DISEASE.

The prevalences of diabetes, coronary heart disease, and hypertension were determined in the survey, and the "one-hour" serum-insulin levels were analyzed in relation to these disease categories. In addition, an attempt was made to identify the "desirable" levels of insulin in groups of subjects selected as being the more healthy, so that the "ideal" ranges could be defined, rather than the "natural" ranges encountered in the total population.

DIAGNOSTIC CRITERIA AND CRUDE PREVALENCE RATES:

(1) CORONARY HEART DISEASE.

The epidemiological diagnosis of coronary heart disease (CHD) depended on positive histories for angina pectoris (AP) or past myocardial infarction (MI) using a modified interpretation of the Rose Questionnaire (Welborn et alii 1969) and also on specific abnormalities of the electrocardiogram (ECG) as classified by the Minnesota Code (Rose and Blackburn 1968).

The category of "probable" CHD comprised all subjects giving positive histories for "probable" AP and/or "probable" MI; and/or having ECG changes of "frank" Q waves, ST-segment and T-wave inversion, complete heart block and complete left bundle branch block (for details see Welborn et alii 1969). "Suspect" CHD subjects had "suspect histories for AP or MI, or borderline ECG changes. All the remaining subjects were classified

as having no CHD. The overall prevalence rate of "probable" CHD was 68 per 1000 for males and 58 per 1000 for females.

(2) HYPERTENSION.

All subjects with systolic pressures exceeding 160 mm. mercury or diastolic pressures exceeding 96 mm. mercury, and subjects giving a history of current treatment for high blood pressure, were labelled hypertensive. There were 485 males and 575 females with "hypertension", giving prevalence rates of 30% and 34% respectively. These stringent criteria for the diagnosis of elevated blood-pressure are arbitrary, but are the same as those selected by Framingham workers as a "risk" category (Dawber et alii 1957). Some further justification for their use is discriminating disease from health derives from studies of non-urbanized peoples (Lovell et alii 1966, Whyte et alii 1958), in whom the occurrence of vascular disease is very low, and the mean blood pressures remain well below these limits, without rising markedly with age.

(3) DIABETES MELLITUS.

The prevalence of "known"diabetes was ascertained by interrogation of respondent subjects. In the publicity campaign preceding the survey, it was made clear that severe diabetics should not take the glucose load, and that patients in doubt should consult their local doctors. Of 48 "known" diabetics, 16 omitted the glucose load and their data was placed on separate files and has not been included in any of the present analyses. Insulin levels were measured on all but 1 of the remaining patients, but 3 additional subjects (one male aged 70, and two females aged 49 and 53) were excluded from the following account of insulin levels in diabetics because it was found that they were receiving insulin therapy, and had evidence suggesting the presence of insulin antibodies (serum-insulin values exceeding 400 μ U/ml.) The 28 "known" diabetics studied represented

a group of previously diagnosed maturity-onset diabetics who had taken the glucose load and were on dietary or oral therapy.

"New" diabetics in the survey were identified by the following means. Of 59 subjects (not known to have diabetes) with "one-hour" blood-sugar levels at or exceeding 200 mg/100ml, 56 attended for follow-up oral glucose tolerance tests under standard conditions, including dietary preparation (200g. carbohydrate daily for 5 days). 29 of these were found to have venous blood-sugar levels of 130 mg/100ml at 120 minutes after 50s. glucose, and were designated "new" diabetics (regardless of age). Insulin levels were measured on all but one of these subjects.

The prevalence of "known" diabetes in the respondent population was 16 per 1000 for males and 12 per 1000 for females; the prevalence of "new" diabetes 7 per 1000 for males and 10 per 1000 for females; and the total prevalence of diabetes 24 per 1000 for males and 22 per 1000 for females. These rates are reported in greater detail (Welborn et alii 1968) where it is shown that the "new" diabetics have an increased frequency of diabetic symptoms, obesity, and manifestations of vascular disease.

RESULTS

THE "ONE-HOUR" SERUM-INSULIN AND DIABETES.

Comparisons of the mean values for age, blood-sugar, percentage desirable weight, and $\log_{10}$ serum-insulin, for the "new" diabetics, "known" diabetics, and the total population, are shown in Table 19. Despite considerable elevation of mean blood-sugar levels and an increase in mean body-weight in the new and known diabetics of both sexes, serum-insulin levels, although somewhat elevated, show very considerable overlap with

the range of values for all males or all females. The small number of diabetic subjects having insulin levels less than the sex- and decade- specific 10th percentiles (see Figure 6) or greater than the 90th percentiles indicates that the discriminating ability of serum-insulin for diabetes is negligible.

The "one-hour" serum-insulin of all non-insulin-requiring diabetics is shown in Table 20 by range of blood-sugar (less than 250 mg/100ml, 250-299 mg/100ml, greater than 299 mg/100ml) and degree of obesity (less than, or greater than 120%). The general tendency for diabetics with blood-sugars less than 300 mg/100ml to have elevated levels of insulin, whether lean or obese, is evident, as compared to the mean level for the whole population, but only achieves statistical significance for obese diabetics with blood-sugar levels 250-299 mg/100ml ($p < 0.01$). Diabetics with blood-sugar levels greater than 299 mg/100ml have normal or less than normal insulin levels. Within the group of obese diabetics, those with blood-sugars of greater than 299 mg/100ml had significantly lower $\log_{10}$ serum-insulin values than those with blood-sugars of 250-299 mg/100ml ($p < 0.05$). No significant differences were obtained in insulin values between lean and obese diabetics in any of the classes of blood-sugar.

FAMILY HISTORY OF DIABETES MELLITUS.

Of non-diabetic subjects, those with a positive family history of diabetes in any first degree relative (parents, siblings, or children) were contrasted with the remaining subjects in terms of the distribution of their insulin and blood-sugar values, and their percentage desirable weights. Using the decade and sex-specific percentiles for these variables, the numbers of subjects with values in the lower 20%, middle 60% and upper 20% ranges were compared (Table 21).

Females giving a positive family history for diabetes showed a significant trend to exhibit "upper range" serum-insulin values compared with the remaining females ($p < 0.01$) and compared with males having a positive family history ($p < 0.05$). The slight tendencies for more subjects with diabetic relatives to be obese, and to have "upper range" blood sugars were apparent, but these were strictly non-significant ($p = 0.3-0.5$). The trend for elevated serum-insulin in females with positive family histories was evident at all ages. (Table 22.)

THE "ONE-HOUR" SERUM-INSULIN AND CORONARY HEART DISEASE.

Subjects with "probable" coronary heart disease (CHD) were older (mean age for males 62, females 63 years), and had higher mean "one-hour" blood-sugar levels (males 123mg/100ml, females 131mg/100ml) than males with no CHD (mean age 47 years, mean blood-sugar 101mg/100ml) and females with no CHD (mean age 46 years, mean blood-sugar 103mg/100ml). There was a small gradient in mean percentage desirable weights for "probable" CHD subjects (males 115%, females 127%) compared with subjects with no CHD (males 112%, females 119%). A difference in serum-insulin was to be anticipated, but this was small, although significant: the mean values for $\log_{10}$ serum-insulin being; -

males	:	no CHD	- - - - -	1.61	(41 μ U/ml)
	:	"probable" CHD	--	1.73	(53 μ U/ml) $p < 0.001$

females	:	no CHD	- - - - -	1.68	(48 μ U/ml)
	:	"probable" CHD	--	1.76	(58 μ U/ml) $p < 0.01$

Analyzing the mean $\log_{10}$ serum-insulin by 10 year age groups (Table 23), it is seen that males aged 30 to 59 years, and females aged 40 to 49 years, tended to have distinctly higher levels; and the differences were significant for both sexes at the age 40 - 49 years ($p < 0.01$). The older subjects

with CHD had very similar insulin values to the "no CHD" groups, as shown in Figure 18. Thus serum-insulin tended to discriminate only the younger subjects with CHD.

Because of the inter-relationships with age, the blood-sugar level, and obesity, the "mean relative prevalence" for serum-insulin was calculated (Table 24) by the method of Newell (1965). In this form of analysis, each of the three variables, serum-insulin, blood-sugar, and percentage desirable weight were classified as being in the "upper range" (U) on the "lower range" (L) using the sex-specific and decade-specific 80th percentile values as the cutting point. The "mean relative prevalence" for a variable is a "weighted" estimate of the "risk ratio" : -

$$\frac{\text{prevalence of "probable" CHD for all persons in the upper range}}{\text{prevalence of "probable" CHD for all persons in the lower range}}$$

over all combinations of ranges of the other particular variables under consideration. The "weighting" procedure involves a variance estimate that takes into account the numbers in the subgroups. A detailed account of this method of analysis, including tests for interaction of the variables, and estimates of significance using the chi-squared test, has been presented (Epstein et alii 1965). The results in Table 24 reveal that in both sexes, the "one-hour" blood-sugar level exerted a significant discrimination, the "mean relative prevalence ratios" being 1.7 for males ($p < 0.05$) and 2.1 for females ($p < 0.01$). The interpretation of these figures is that males and females in the population having "upper range" blood-sugar levels had 1.7 and 2.1 times (respectively) the prevalence rate of CHD compared with subjects having "lower range" blood-sugar levels, holding constant the effects of obesity and serum-insulin. For serum-insulin, in males, the

"mean relative prevalence ratio" of 1.3 was the same as for degree of obesity, and was not significant. Females with "upper range" serum-insulin had less CHD than the remaining females ("mean relative prevalence ratio" of 0.6) thus the conventional cutting point failed absolutely to discriminate CHD. The "mean relative prevalence ratio" for percentage desirable weight in females was 1.7 ($p < 0.05$) but this showed interaction with blood-sugar as a risk-factor.

The crude risk-ratios in 10 year age groups for elevated serum-insulin (upper 20%) was examined as shown in Table 25. The crude risk-ratio for a variable is defined as: -

prevalence of "probable" CHD for all persons in
the upper range

prevalence of "probable" CHD for all persons in
the lower range

without the "weighting" procedure employed in the calculation of "mean relative prevalence ratios" and without taking into account the influence of other variables. None of the risk-ratios achieved statistical significance by the chi-squared test, although in the younger males (for whom the numbers of CHD cases were small) there was the suggestion that a study of larger numbers might yield better discrimination. However, when the "mean relative prevalence ratios" for serum-insulin, blood-sugar and percentage desirable weight were calculated for males aged 30 - 49, the values were: -

<u>"mean relative prevalence (U/L)" for males aged 30-49</u>	
blood-sugar	3.4 ($p < 0.05$)
serum-insulin	2.6 (N.S. - $p < 0.10$)
percent desirable weight	1.4 (N.S.)

Further analyses showed that serum cholesterol and serum uric acid had even greater risk ratios than blood-sugar in this age group. Thus, even in younger males, the "one-hour"

serum-insulin level failed to be a useful discriminating factor for CHD prevalence. The extent of this failure, in all ages is shown in Table 26, where the crude risk-ratios of various other factors are shown. Compared with the significant "predictive" power of blood pressure, serum cholesterol, and other variables, serum-insulin ranks as the least useful.

THE "ONE-HOUR" SERUM-INSULIN AND HYPERTENSION.

Hypertension was defined as a systolic blood pressure exceeding 160 mm.Hg. and/or a diastolic pressure greater than 96 mm. Hg. The "one-hour" $\log_{10}$ serum-insulin values were found to be significantly higher in hypertensive males aged 30 to 69, and in hypertensive females aged 40 to 49 (Table 27). Figure 19 shows that the mean values for serum-insulin rose more steeply with age in the hypertensive as compared with the non-hypertensive subjects. Since multiple regression analysis had failed to reveal any independent association of blood pressures with serum-insulin, the differences observed are almost certainly mediated by other variables, including blood-sugar levels, percentage desirable weight, and serum uric acid, with which both systolic and diastolic pressures were strongly correlated.

Because of the provocative hypothesis (Conn 1965) that potassium deficiency in hypertension caused impaired insulin release with resulting hyperglycaemia, a specific analysis was made of the inter-relationships of blood pressure and serum-potassium with the "one-hour" blood-sugar and serum-insulin levels. Using again the age- sex- specific percentiles, subjects were classified as having "upper range" (U) or "lower range" (L) systolic blood pressures using the 80th percentile divisions; and as having "lower range" (L) or "upper range" serum-potassium levels using the 20th percentile divisions (3.9-4.1 mEq/l). The mean blood-sugar and $\log_{10}$ serum insulin

values were calculated for the various combinations. In males (Table 28 and Figure 20) the tendency for hypertensive subjects to have elevated blood-sugar levels was apparent, and in particular, males with hypertension and low serum-potassium showed some evidence for more severe hyperglycaemia. More striking differences were obtained with the mean $\log_{10}$ serum-insulin values. Hypertensive males with relative hypokalaemia had distinctly elevated insulin values compared with normokalaemic hypertensives and with the non-hypertensive subjects. The differences in $\log_{10}$ serum-insulin between the hypertensive subjects with low serum-potassium, and the normotensive subjects with normal (i.e. "upper range") potassium levels, were significant for males aged 50 - 59 ($p < 0.01$) and 60 - 69 ($p < 0.05$), but at the age of 70+ years, no such trend was evident. (Table 28). The females (Table 29) showed a much less consistent tendency for hypertensive subjects to have hyperglycaemia, and the range of mean $\log_{10}$ serum-insulin values was more narrow, with no similar evidence for hypokalaemic hypertensives to show elevated serum-insulin.

The data suggests that in males with hypertension and coexisting low serum-potassium levels, hyperglycaemia occurs in the presence of elevated levels of circulating insulin. Hypertensive males with normal levels of serum-potassium have hyperglycaemia but almost normal levels of serum-insulin. The available numbers do not allow further dissection of the data. Analysis of the diastolic pressures in a similar manner revealed less convincing trends.

THE "ONE-HOUR SERUM-INSULIN IN HEALTH.

The effect of activity status was first examined. Although no good index of exercise assessment has yet been devised, occupational categories and rural-urban differences, in males, should provide clues to the possible metabolic dispositions of the sedentary versus the active. Therefore

males classified by occupation * as "professional, administrative, executive" and as "clerical, sales" personnel, were designated sedentary, and those classified as "farmers, outdoor workers" and "labourers, including farm labourers" were grouped as active. Other occupational categories were not considered because the activity status was ambiguous. The mean values for $\log_{10}$ serum-insulin, blood-sugar, and percentage desirable weight, of active versus sedentary males by age, is shown in Table 30 and Figure 21. Sedentary males had a constant elevation of serum-insulin levels compared with active males, and the differences were significant at the ages 20 - 29 years ($p < 0.05$) and 40 - 49 years ($p < 0.05$). The progressive and gradual rise of serum-insulin in the sedentary was in contrast with the steady levels in active subjects under 50 years, but thereafter the mean values converge. No significant differences were seen in the one-hour blood-sugar levels nor the percentage desirable weights of the two groups (Table 30). A similar contrast in serum-insulin levels could be seen (Figure 22) in the younger urban males (high levels) compared with rural males (low levels) but the differences were much less marked and were non-significant.

Finally, in an attempt to ascertain the "ideal" levels of serum-insulin under the conditions of this study, groups of males and females were defined by excluding the obese (males with percentage desirable weight $> 120\%$, females $> 130\%$), the hyperglycaemic (all diabetics, and subjects under 45 years with "one-hour" blood-sugar levels of less than 185 mg/100ml); all remaining subjects with blood-sugars less than 200 mg/100ml), and all those with vascular diseases (survey diagnosis of "probable" coronary heart

* Australia Bureau of Census and Statistics Classification

disease hypertension or claudication). The mean insulin values, and the range of ± 2 standard deviations, by age, are shown in Table 31(A). The lower values for $\log_{10}$ serum-insulin showed no rise with age until 60 years in males and 50 years in females. A wide (eight to tenfold) range of absolute levels continued to be evident in each age group.

Figure 23 contrasts the age trends of serum-insulin in the "ideal" groups, the total population, hypertensive subjects, and those with coronary heart disease. In comparison with the "ideal" subjects, those with vascular disease have a substantial and premature rise of insulin levels, particularly in males. The "one hour" serum-insulin, in these groups, would appear to provide an index of vascular ageing, and the trend of the total population, intermediate between the vascular disease and the "ideal" groups, suggests considerable latent disease in the community.

DISCUSSION

In epidemiological studies of this nature, a responsibility exists to ascertain the extent to which biochemical variables are associated with disease; both from the point of view of identifying segments of the community at greater risk of having or developing clinical illness, but also in discerning general trends that may assist in interpreting the pathogenesis or mechanism of disease, as a guide for further research. For example, the "one hour" blood-sugar level in the Busselton study was very useful in screening for undetected diabetes, and showed a 50% sensitivity in finding cases subsequently proved to have abnormal glucose tolerance tests by conventional criteria. An elevated "one hour" blood-sugar level was also found to be a significant independent "risk factor" associated with prevailing coronary heart disease, and this suggests its possible application in

identifying subjects having future risk of the disease. Similar observations (Keen et alii 1965; Ostrander et alii 1965) have recently revived much interest in the possible aetiological role of impaired carbohydrate metabolism in vascular disease. In contrast with the efficacy of blood-sugar measurements, the "one hour" serum-insulin levels have proved to be of limited use in discriminating disease, but have nevertheless yielded additional information of possible value in clarifying concepts of the mechanism of disease.

The diabetic group studied were maturity-onset type of mild to moderate severity. The data of those requiring insulin injections were excluded from the results. With the small numbers of cases, and the wide scatter of insulin values, few of the apparent differences found in diabetics achieved statistical significance; and the 10th and 90th age and sex-specific percentile values for the serum-insulin levels of the general population provided no useful discrimination of diabetic subjects. The tendency for less severe diabetics with "one hour" blood-sugar levels of less than 300 mg/100 ml to have higher mean insulin levels than normal probably reflects their greater mean age (+ 15-20 years) and greater obesity (+ 15% percentage desirable weight) than the average population; and these general trends are in accord with the independent contribution of such factors as described by multiple regression analysis in the previous section. Similarly, the mean serum-insulin of diabetics with blood-sugars exceeding 300 mg/100 ml was lower than the less severe diabetics (but within the "normal" range), and indicates the known association of insulin lack with more marked hyperglycaemia. It must be stressed, nevertheless, that the multiple regression analysis defined trends for the population as a whole, and to identify, within a diabetic group, the independent contributions of such factors with accuracy would

require substantially greater numbers. That "moderate" diabetics have elevated serum-insulin levels, and "severe" diabetics comparatively low values, has been shown in previous studies (Berson et alii 1965; Karam et alii 1965).

The insulin levels of first degree relatives of diabetics were examined in the hope that some clue would emerge as to the insulin status of possibly pre-diabetic subjects. This area of research has produced conflicting results. Clinical studies of normoglycaemic individuals having genetic predisposition to diabetes, that is with both parents or an identical twin sibling diabetic, have revealed (1) no clear difference in serum-insulin levels during oral glucose tolerance tests (Karam et alii 1965); (2) an impaired insulin response to oral glucose (Pyke et alii 1967; Taylor et alii 1967; Soeldner et alii 1968) or to sustained intravenous hyperglycaemia (Cerasi and Luft 1967); and finally (3) elevated levels of insulin-like activity (Steinke et alii 1963). Other groups of subjects considered to have greater risk of diabetes are normoglycaemic glycosuric individuals, and these tend to have an excessive insulin response (Hales et alii 1968). A preliminary report of a prospective study of chemical diabetics (Danowski 1969) also indicates that in the evolution of frank clinical diabetes, increased hyperinsulinism is the more consistent finding. In the Busselton data, subjects with a first degree relative known to be diabetic showed no tendency at all to have low serum-insulin levels. However females (of all ages) with positive family histories tended to have insulin values in the upper range, and this trend was statistically significant. While this adds some positive evidence to one side of the controversy reviewed above, two qualifications are necessary. First, it is obvious that prospective studies are essential to confirm any of the hypotheses about the "predictive" ability of high or low

insulin levels for diabetes, and these are in progress. Second, it appears likely that there are two distinct components of the pathogenesis of diabetes, and each requires separate study. "Insulin resistance", as manifest by elevations of serum-insulin, is partially identified by the "one hour" glucose tolerance test, although more definitive methods of identifying this are very desirable. Impaired pancreatic secretion could be an entirely separate phenomenon with different patterns of inheritance and pathogenesis, and the more provocative tests of secretory response are probably of greater value in unmasking this trait.

Coronary heart disease subjects, identified by standard epidemiological criteria, had significantly elevated "one hour" serum-insulin levels when compared with the large segment of the population showing no evidence of the disease. When age was taken into account, significant hyperinsulinism existed in males aged 40-49, and females aged 40-49 years, but older age groups had almost identical mean serum-insulin levels. Insulin values were of little use in discriminating established heart disease cases as compared with blood pressure, serum cholesterol, or the "one hour" blood-sugar level. The extensive cross-classifications and other analyses that have been presented show that serum-insulin as an epidemiological "risk factor" for screening purposes is unlikely to be of value; but it must be emphasized that this is an entirely separate issue from its application in identifying possible aetiological mechanisms in vascular disease.

The efficacy of elevated serum cholesterol and blood pressure levels in identifying subjects with considerably greater risk of developing coronary incidents is unequivocal and has been confirmed by numerous prospective studies. The "risk" identified by these factors is comparatively short-term, and it seems possible indeed that their predictive

power is high because the risk factors themselves represent the concomitants of already established vascular disease. The two major processes known to be implicated in coronary heart disease are atherosclerosis, and thrombosis; yet years of research have failed to establish convincing aetiological hypotheses for the primary causal role of hypercholesterolaemia, or hypertension, in these phenomena.

It is proposed that insulin is an important aetiological agent in the early development of atherosclerosis. Clinical reports of subjects with myocardial infarction (Sowton 1962; Cohen and Shafrir 1965) suggested that impaired carbohydrate tolerance was common with this condition and that this may reflect some underlying metabolic abnormality. Population studies (Epstein et alii 1965; Keen et alii 1965; Ostrander et alii 1965) provided substantial confirmation that hyperglycaemia and many manifestations of vascular disease were associated. In pursuing the mechanism of hyperglycaemia in cardiovascular disease, further clinical studies revealed that, even when glucose tolerance was normal, consistent elevations of serum-insulin levels were found in coronary heart disease (Peters and Hales 1965; Nikkila et alii 1965; Tzagournis et alii 1967); also in essential hypertension, and in peripheral vascular disease (Welborn et alii 1966). The present report provides the first data from a full-scale epidemiological study, and confirms that in the general population, a significant association can be found between high serum-insulin and coronary heart disease; specifically in subjects with "premature" disease, under the age of 50 (Figure 18). The failure to show clear associations in young adults may reflect lack of numbers of disease cases. Also there are some reservations about the validity of diagnostic criteria (e.g. T wave inversions on ECG), in the young, although subjects with identifiable rheumatic and other forms

of heart disease were excluded from the diagnosis of coronary heart disease (Welborn et alii 1969). Although serum-insulin provided no effective biochemical discrimination for coronary heart disease, a finding that will clearly disconcert those who relate aetiological processes to the strength of statistical associations found in populations (for example, Stamler 1968), such attitudes need challenging. Insulin as an early initiation of the atherosclerotic process should not be expected to identify subjects developing vascular occlusion, which is a late or end-stage phenomenon of coronary heart disease, and may well be produced by other factors including thrombosis. Atherosclerosis becomes almost universal with increasing age in Western communities (Mitchell and Schwartz 1965) and the distinction between those with the disease plus clinical evidence of major vascular occlusion, and the remainder of the population, is likely to be poorly defined. In addition, the wide range of insulin levels encountered in man (partly a reflection of obscure "insulin resistance" which itself may prove to be of aetiological significance) certainly impedes the value of serum-insulin measurement as a screening factor for the identification of short-term risk.

Some support for this hypothesis has emerged from animal and laboratory studies. In experimental fowl atherosclerosis induced by lipid feeding, insulin administration prevented the rapid regression of coronary atheromata that occurred with control fowls, when the diet was ceased (Stamler et alii 1955). In similar studies insulin had an occasional but inconsistent action in antagonizing the protective effect of oestrogens. No direct atherogenic influence of insulin administration in fowls was evident, but the experiments were of short duration, lasting several weeks (Stamler et alii 1960). Alloxanized rabbits, presumed to be insulin-deficient, developed lesser degrees of atheroma than control animals on

an atherogenic diet (Duff and McMillan 1949; McGill and Holman 1949). In the laboratory, "in vitro" studies of endothelial tissue are yielding more direct evidence of a causative relationship between insulin and early atheroma. Vascular endothelium is less sensitive than adipose tissue to insulin, but it shows similar metabolic responses by converting labelled glucose to lipid and by inhibiting lipolysis and arterial tissue lipase (Mahler 1964, 1966). The recent studies of Stout (1968) show that insulin has an acute "in vivo" action, in promoting lipid and specifically cholesterol synthesis of rat aortic tissue.

Atherosclerosis is characterized by both the intracellular and the extracellular accumulation of lipid in endothelial tissue (Mitchell and Schwartz 1965). At present, insulin appears to be implicated in the former process, at least. This biological action of insulin provides a more feasible explanation for endothelial lipogenesis than the passive filtration of circulating lipids or other more obscure phenomena proposed by those who support the aetiological role of hyperlipidaemia. The adverse effects of inappropriate diet, obesity, and inactivity may well be mediated by insulin. Proof of this hypothesis is important, and requires further detailed knowledge of vascular tissue pathophysiology; and prolonged prospective observation of human populations. A more clear understanding of the pathogenesis of vascular disease is vital for the correct orientation of preventive regimes in public health programmes of the future: lowering the levels of serum-insulin may prove to be an important aim in preventing vascular disease, both in diabetics and in the general population.

Subjects with hypertension, assessed by rather stringent diagnostic levels of blood pressure, showed consistent elevations of the "one hour" serum-insulin level in all but elderly subjects. Hypertensive subjects were also substantially more hyperglycaemic and more obese, and this may account for the lack of any independent association between systolic or diastolic blood pressure and $\log_{10}$ serum-insulin by multiple regression analysis. Nevertheless, the higher serum-insulin levels of hypertensive subjects, and the accelerated rise, with age, of serum-insulin levels, suggests a possible mechanism whereby hypertension promotes atherosclerosis. The cause of "insulin resistance" in hypertensive subjects may relate to impaired permeability of small blood-vessels, as has been discussed.

Conn, in 1965, suggested a provoking hypothesis for the association between hypertension and diabetes. On the basis of a large experience in primary aldosteronism, he postulated that hyperglycaemia in hypertensive subjects was very commonly linked with potassium deficiency; and that the latter was due to unsuspected hyperaldosteronism. His anecdotal accounts indicated that the impaired glucose tolerance of such potassium deficient patients was due to defective insulin secretion, which was promptly reversed by potassium loading. It was surprising, perhaps, to find some suggestive trends in the Busselton study linking hypertension, hyperglycaemia, and potassium deficiency (Figure 20). Males with hypertension and low serum potassium levels showed higher blood-sugar levels with higher serum-insulin levels. Thus the mechanism of hyperglycaemia in these subjects appears to relate more to insulin insensitivity than to impaired insulin release. (The greater proportion of "hypertensive" subjects were not receiving therapy and no differences in blood pressure levels of normokalaemic and hypokalaemic subjects were found.)

Potassium depletion causes impaired glucose tolerance in normal subjects (Sagild et alii 1961) but it would appear that it is more potently diabetogenic in hypertensives. This preliminary finding clearly requires more detailed exploration, to further evaluate the meaning of low serum potassium in "essential" hypertension, and its contribution to hyperglycaemia.

"Ideal" subjects in the population continued to have a tenfold range of absolute insulin values, as expected, perhaps, from the range obtained with healthy volunteers previously studied. However, with obesity, hyperglycaemia, and vascular disease excluded, the remaining subjects in the community showed no rise of serum-insulin until 60 years in males and 50 years in females. This is in sharp contrast with the accelerated or premature rise, with age, observed in the obese, the hyperglycaemic, and those with vascular disease. Further support for the concept that "steady" insulin levels with increasing age are in general indication of health derives from the finding that "active" as compared to "sedentary" males show a trend of serum-insulin with age similar to the "ideal" subjects. In epidemiological comparisons of primitive versus urban communities, and of active versus sedentary peoples, the measurement of serum-insulin, together with other biochemical variables, may provide a useful indication of metabolic status in relationship to the common diseases of "civilization".

SUMMARY

I.

In an epidemiological study of a rural community, serum-insulin levels were assayed from 3,313 adults approximately one hour after a 50 g. oral glucose load. The advantages of obtaining data from large numbers of subjects outweighed the disadvantages of the comparatively uncontrolled conditions of the study. The log-normal distribution of serum-insulin levels was confirmed, and no good evidence for the existence of sub-populations of high or low insulin secretors was obtained. Females had consistently higher levels than males. In both sexes, the "one hour" serum-insulin levels rose moderately but unequivocally with age. A large number of physical and biochemical measurements were found to be related to the $\log_{10}$ serum-insulin values, and the nature of their independent associations with insulin was determined by multiple regression analysis. The "one hour" blood-sugar level showed the most strong independent association with insulin values. A steep positive correlation between insulin and blood-sugar levels less than 180 mg/100 ml existed, implying that "insulin resistance" was responsible for mild degrees of hyperglycaemia. With "one hour" blood-sugar levels greater than 180 mg/100 ml, declining insulin values occurred and indicated that impairment of pancreatic secretory response related to the more severe hyperglycaemia. Age beyond 50 years, excessive body weight, and elevated serum uric acid were independently associated with a tendency for high serum-insulin levels. In both sexes, the time of the test in relation to last taking food, was found to be a substantial source of variation of insulin response. Apart from high serum-insulin levels in relation to meal times, no innate diurnal rhythm of

insulin release could be discerned. The insulin levels were inversely and independently related to height; thus the insulin response to a standard glucose load varies with an index of lean body mass. In females, increasing plasma osmolality and declining insulin levels were found to be associated independently to other factors: it is suggested, with caution, that hyperosmolality is a possible factor in the impaired secretory response of the pancreas with more severe diabetes. About one-third of the variance of $\log_{10}$ serum-insulin was attributable to its relationship with other factors measured. The meaningful trends that have emerged support the validity of insulin levels measured by immunoassay.

II.

As a screening test for the identification of subjects with disease, the measurement of the "one hour" serum-insulin level was of little practical value, but the general trends discerned gave some useful insights into possible pathological mechanisms.

Mild maturity-onset diabetics had somewhat higher mean insulin levels, at one hour, than the general population; whereas those with diabetes of moderate severity had values essentially in the normal range. Using the 10th and 90th age-specific percentile insulin levels of the population, diabetic subjects showed no tendency to segregate in the upper or lower ranges. However, non-diabetic females with a close family history of diabetes showed more than the expected number of subjects with high insulin levels, and this was consistent at all ages. The significance of this finding must await prospective observation, but it is possible that hyper-insulinism will prove to be a recognizable "pre-diabetic" phenomenon.

Subjects with a survey diagnosis of "probable" coronary heart disease showed elevated mean serum-insulin levels at "one hour", as compared with those with no coronary heart disease. This trend was essentially due to the higher mean values of males aged 30-59 years and females aged 40-49 years. This finding is compatible with the hypothesis that insulin may be an early aetiological factor in the genesis of atherosclerosis. Further evidence supporting this theory is reviewed.

Hypertensive subjects in the population had elevated mean serum-insulin levels and showed an accelerated rise of insulin values with age. No independent association between $\log_{10}$ serum-insulin and blood pressure levels was found, but the hypertensive subjects tended to be more obese and more hyperglycaemic, and these factors could have accounted for their hyperinsulinism. Insulin may initiate atherosclerosis in hypertensive subjects. Males with high systolic blood pressures but low serum potassium levels showed increased insulin resistance: further studies of these inter-relationships are indicated.

The mean insulin values of "ideal" subjects who were lean, normoglycaemic and with no overt vascular disease were found to stay level with age, with no rise until 60 years in males, and 50 years in females. Males with active occupations had a similar pattern. In general, therefore, low insulin levels provide an index of health. For epidemiological comparisons of population samples, the "one hour" serum-insulin levels may give an additional and useful indication of metabolic or vascular disease.

TABLE 19.

The "one-hour" serum-insulin and diabetes: comparison of the mean values for age, blood-sugar, percentage desirable weight, and $\log_{10}$ serum-insulin, of 24 new diabetics, 32 known diabetics (not insulin dependent), and all males and females. The standard deviation of the $\log_{10}$ serum-insulin values is also given. The number of diabetic subjects with insulin levels outside the decade-specific and sex-specific 10th and 90th percentile values of the population are shown.

population are shown						
SUBJECTS AND NUMBER	MEAN AGE (yrs)	MEAN BLOOD- SUGAR (mg/ 100ml.)	MEAN PER- CENT DESIR- ABLE WEIGHT (%)	MEAN "ONE-HOUR" LOG ₁₀ SERUM- INSULIN (± 1 S.D.	NO. OF SUBJECTS WITH S. INSULIN OUTSIDE PER- CENTILES: *	
					<10th	>90th
<u>MALES</u>						
New diabetics (12)	63	285	127	1.88±0.34	0	3
Known diabetics(16)	64	198	124	1.74±0.23	0	2
All males (1629)	47	103	110	1.63±0.28	-	-
<u>FEMALES</u>						
New diabetics (16)	68	239	135	1.78±0.33	3	2
Known diabetics(12)	69	234	133	1.76±0.35	3	3
All females (1684)	46	105	120	1.69±0.26	-	-

* Sex-specific, decade-specific percentiles.
(see Figure 6.)

TABLE 20.

Diabetic subjects (new and known): mean $\log_{10}$ serum-insulin ± 1 standard error by range of blood-sugar (<250; 250-299; >299mg/100ml.) and range of percentage desirable weight (<120%; >120%).

ALL DIABETIC SUBJECTS (N = no.).	"ONE-HOUR" BLOOD-SUGAR LEVELS		
	LESS THAN 250mg/100ml.	250 TO 299mg/100ml.	GREATER THAN 299mg/100ml.
<u>OBESE:</u>			
Mean $\log_{10}$ S.Insulin (± 1 S.E.):	1.82(± 0.10)	^{**} 2.05(± 0.13)	1.56(± 0.09)
Mean percent weight:	155%	151%	151%
	(N = 10)	(N = 6)	(N = 5)
<u>LEAN:</u>			
Mean $\log_{10}$ S.Insulin (± 1 S.E.):	1.71(± 0.05)	1.95(± 0.15)	1.73(± 0.08)
Mean percent weight:	117%	112%	120%
	(N = 23)	(N = 8)	(N = 4)

ALL MALES AND FEMALES

Mean $\log_{10}$ S.Insulin ± 1 S.E. : 1.655 $\pm$ 0.006 (N = 3313)

TABLE 21. Positive family history of diabetes by ranges of the "one-hour" serum insulin, the blood-sugar level, and the percentage desirable weight. The number of subjects in the age - and sex - specific percentile ranges <20th; 20th - 80th; >80th for each variable are shown. 59 diabetic subjects are excluded from the analysis.

FAMILY HISTORY OF DIABETES	NUMBER OF SUBJECTS IN PERCENTILE RANGES FOR			χ^2 (d.f.) (=2)
	SERUM-INSULIN			
<u>MEN</u>	<20th	20 - 80th	>80th	
negative	276 (20%)	822 (59%)	285 (21%)	2.0
positive	44 (20%)	137 (63%)	36 (17%)	(N.S.)
<u>WOMEN</u>				
negative	292 (21%)	830 (60%)	269 (19%)	10.4
positive	35 (13%)	162 (62%)	66 (25%)	(p<0.01)
BLOOD SUGAR				
<u>MEN</u>	<20th	20 - 80th	>80th	
negative	267 (19%)	854 (62%)	262 (19%)	1.7
positive	45 (21%)	124 (57%)	48 (22%)	(N.S.)
<u>WOMEN</u>				
negative	269 (19%)	857 (62%)	265 (19%)	1.8
positive	52 (20%)	152 (58%)	59 (22%)	(N.S.)
PERCENT DES. WEIGHT				
<u>MEN</u>	<20th	20 - 80th	>80th	
negative	269 (20%)	846 (61%)	268 (19%)	2.9
positive	36 (17%)	129 (59%)	52 (24%)	(N.S.)
<u>WOMEN</u>				
negative	264 (19%)	854 (61%)	273 (20%)	1.8
positive	46 (18%)	156 (59%)	61 (23%)	(N.S.)

TABLE 22.

Positive family history of diabetes in non-diabetic females: the trend for elevated serum-insulin is consistent at all ages.

<u>FEMALES</u>				
FAMILY HISTORY OF DIABETES	AGE (years)	SERUM-INSULIN: number of subjects in the percentile range:		χ^2 (d.f.) (=5)
		<20th	>80th	
Negative	21 - 39:	104	96	12.6
	40 - 59:	119	107	
	60+ :	69	66	
Positive	21 - 39:	13	24	(p<0.05)
	40 - 59:	16	28	
	60+ :	6	13	

TABLE 23. The "one hour" serum-insulin and coronary heart disease (CHD): mean $\log_{10}$ serum-insulin levels are given for males and females having no CHD, "suspect" CHD, and "probable" CHD in 10 year-age groups.

MALES: MEAN $\log_{10}$ SERUM-INSULIN (and no. of subjects)			
AGE (years)	NO CHD	"SUSPECT" CHD.	"PROBABLE" CHD.
21 - 29:	1.53 (243)	1.52 (11)	1.37 (1)
30 - 39:	1.56 (261)	1.53 (14)	1.71 (4)
40 - 49:	1.59 (306)	1.51 (24)	1.80 ** (15)
50 - 59:	1.62 (270)	1.75 (18)	1.72 (25)
60 - 69:	1.72 (206)	1.83 (20)	1.71 (38)
70+ :	1.75 (130)	1.79 (15)	1.73 (28)

FEMALES: MEAN $\log_{10}$ SERUM-INSULIN (and no. of subjects)			
AGE (years)	NO CHD	"SUSPECT" CHD.	"PROBABLE" CHD.
21 - 29:	1.63 (259)	1.72 (11)	2.42 (1)
30 - 39:	1.62 (302)	1.63 (12)	1.57 (6)
40 - 49:	1.64 (325)	1.66 (25)	1.79 ** (5)
50 - 59:	1.69 (279)	1.70 (21)	1.71 (20)
60 - 69:	1.79 (193)	1.78 (23)	1.78 (29)
70+ :	1.84 (113)	1.83 (24)	1.79 (36)

** $p < 0.01$ (compared with the "no CHD" group)

TABLE 24. "Probable" coronary heart disease prevalence by ranges of the "one-hour" serum-insulin, blood-sugar level (BSL) and percentage desirable weight (%DW), and the "mean relative prevalence" values for the upper versus the lower ranges of these variables. U = above, and L = at or below the age - and sex - specific 80th percentile values.

MEN (21 YEARS AND OLDER : N = 1629)						
INSU- LIN	BSL	% DW	CHD (No.)	TOTAL (No.)	PRE- VAL- ENCE PER 1,000	MEAN RELATIVE PREVALENCE (U/L) FOR:
L	L	L	48	895	54	
L	L	U	12	175	69	
L	U	L	18	192	94	Blood-sugar:1.7 (p<0.05)
U	L	L	8	131	61	Serum-insulin: 1.3 (N.S.)
L	U	U	5	52	96	% Desirable weight: 1.3 (N.S.)
U	L	U	8	83	96	
U	U	L	7	66	106	
U	U	U	5	35	143	
All Males			111	1629	68	
WOMEN (21 YEARS AND OLDER : N = 1684)						
L	L	L	46	906	51	
L	L	U	11	200	55	
L	U	L	14	199	70	
U	L	L	6	162	37	Blood-sugar: 2.1 (p<0.01)*
L	U	U	10	42	238	Serum-insulin: 0.6 (N.S.)
U	L	U	2	67	30	% Desirable weight:1.7 (p<0.05)*
U	U	L	4	71	56	
U	U	U	4	37	108	
All Females			97	1684	58	

* Interaction (p ≈ 0.05)

TABLE 25. Prevalence of coronary heart disease (CHD) by range of serum-insulin: "U" = above, and "L" = at or below the age- and sex- specific 80th percentile values (Figure 6.) Crude risk-ratios in 10 year age groups are shown.

AGE (years)	NO CHD		CHD		RISK-RATIO* FOR ELEVA- TED SERUM- INSULIN
	NO. OF SUBJECTS WITH SERUM-INSULIN IN THE RANGE:		NO. OF SUBJECTS WITH SERUM-INSULIN IN THE RANGE:		
	U	L	U	L	
MALES:					
21 - 29:	50	204	0	1	0
30 - 39:	54	221	2	2	4.1
40 - 49:	63	267	6	9	2.8
50 - 59:	58	230	5	20	1.0
60 - 69:	43	183	10	28	1.5
70+ :	19	126	5	23	1.4
ALL MALES:	287	1231	28	83	1.4
FEMALES:					
21 - 29:	53	217	1	0	(4.1)
30 - 39:	63	251	1	5	0.8
40 - 49:	70	280	1	4	1.0
50 - 59:	62	238	2	18	0.4
60 - 69:	45	171	4	25	0.6
70+ :	28	109	7	29	0.9
ALL FEMALES:	321	1266	16	81	0.8

* Risk-ratio = $\frac{\text{U/L for "CHD"}}{\text{U/L for "no CHD"}}$

TABLE 26.

"Risk-factors" associated with the prevalence of coronary heart disease in Busselton 1966. The risk-ratios indicate the relative frequency of coronary heart disease in subjects with "upper range" values for each factor. The age - and sex - specific 80th percentile levels separate the "upper" from the "lower range".

CORONARY HEART DISEASE.

<u>FACTOR</u>	<u>RISK-RATIO (all ages)</u>	
	<u>MALES</u>	<u>FEMALES</u>
BLOOD-PRESSURE	2.5 (p<.001)	2.0 (p<.01)
SERUM CHOLESTEROL	2.2 (p<.001)	1.6 (p<.05)
SERUM URIC ACID	2.2 (p<.001)	1.3 (N.S.)
"ONE-HOUR" BLOOD-SUGAR	1.7 (p<.05)	1.7 (p<.05)
% IDEAL WEIGHT	1.6 (p<.05)	1.5 (N.S.)
"ONE-HOUR" SERUM-INSULIN	1.4 (N.S.)	0.8 (N.S.)

TABLE 27. The "one-hour" serum-insulin and hypertension: mean $\log_{10}$ serum-insulin levels are given for males and females without hypertension, and with hypertension (systolic blood pressure >160 and/or diastolic blood pressure >96).

MALES: MEAN $\log_{10}$ SERUM-INSULIN (and number of subjects)			
AGE (years)	NO HYPERTENSION	HYPERTENSION	SIGNIFICANCE
21 - 29	1.53 (219)	1.53 (40)	
30 - 39	1.55 (238)	1.60 (41)	$p < 0.01$
40 - 49	1.57 (270)	1.65 (75)	$p < 0.01$
50 - 59	1.61 (220)	1.70 (93)	$p < 0.01^*$
60 - 69	1.71 (130)	1.75 (134)	$p < 0.05$
70+	1.74 (71)	1.76 (102)	
FEMALES: MEAN $\log_{10}$ SERUM-INSULIN (and number of subjects)			
AGE (years)	NO HYPERTENSION	HYPERTENSION	SIGNIFICANCE
21 - 29	1.63 (254)	1.62 (17)	
30 - 39	1.62 (280)	1.66 (40)	
40 - 49	1.63 (265)	1.69 (90)	$p < 0.05^*$
50 - 59	1.67 (172)	1.73 (148)	
60 - 69	1.75 (105)	1.81 (140)	
70+	1.83 (33)	1.83 (140)	

* Variance ratio significant.

TABLE 29.

Mean "one-hour" blood-sugar levels and $\log_{10}$ serum-insulin for females according to range of systolic blood pressure and serum potassium (serum K^+). For each decade, the 80th percentile divided upper range ("U") from lower range ("L") systolic blood pressures; and the 20th percentile divided lower range from upper serum potassium levels.

1684 FEMALES

SERUM K^+	{U = upper 4/5} {L = lower 1/5}	U	L	U	L
SYSTOLIC B.P.	{L = lower 4/5} {U = upper 1/5}	L	L	U	U
Proportion of total subjects:		61%	21%	12%	6%
AGE (years)	MEAN BLOOD-SUGAR LEVEL (mg/100ml.)				
21 - 29:		83	88	89	97
30 - 39:		86	104	99	108
40 - 49:		101	102	105	121
50 - 59:		103	114	118	107
60 - 69:		119	117	132	120
70+ :		128	123	144	122
	MEAN $\log_{10}$ SERUM-INSULIN				
21 - 29:		1.63	1.62	1.63	1.71
30 - 39:		1.59	1.67	1.71	1.65
40 - 49:		1.62	1.68	1.69	1.66
50 - 59:		1.66	1.73	1.78	1.73
60 - 69:		1.77	1.76	1.87	1.77
70+ :		1.85	1.81	1.82	1.88

TABLE 30. Serum-insulin and activity status - (A) Mean "one-hour" $\log_{10}$ serum-insulin; and (B) Mean "one-hour" blood-sugar levels and mean percentage desirable weight; for males by occupational categories classified as "sedentary" or "active".

(A) MALES						
AGE (years)	MEAN "ONE-HOUR $\log_{10}$ SERUM INSULIN		(Signifi- cance)			
	ACTIVE (and number of subjects)	SEDENTARY (and number of sub- jects)				
21-29:	1.53	(110)		1.60	(69)	(p<0.05)
30-39:	1.56	(120)		1.64	(57)	(N.S.)
40-49:	1.56	(158)		1.66	(73)	(p<0.05)
50-59:	1.63	(174)		1.69	(38)	(N.S.)
60-69:	1.72	(85)		1.75	(36)	(N.S.)

(B) MALES						
AGE (years)	MEAN "ONE HOUR" BLOOD-SUGAR (mg/100ml)		(Signif- icance)	MEAN PERCENTAGE DESIRABLE WEIGHT		(Signif- icance)
	ACTIVE	SEDEN- TARY		ACTIVE	SEDEN- TARY	
21-29:	86	91	(N.S.)	107	111	(N.S.)
30-39:	96	92	(N.S.)	111	110	(N.S.)
40-49:	96	102	(N.S.)	114	113	(N.S.)
50-59:	107	122	(N.S.)	113	117	(N.S.)
60-69:	115	108	(N.S.)	115	115	(N.S.)

TABLE 32. (A) Mean "one-hour" $\log_{10}$ serum-insulin, and range of mean $\pm$ 2 standard deviations; (B) mean "one-hour" blood-sugar level and percentage desirable weight: for "ideal" subjects with obesity, vascular disease, and frank hyperglycaemia excluded.

"IDEAL SUBJECTS"

(A)

AGE (years)	<u>"ONE-HOUR" $\log_{10}$ SERUM- INSULIN</u>		<u>ABSOLUTE INSULIN VALUES</u>	
	MEAN (and no. of subjects)	RANGE OF MEAN $\pm$ 2 S.Ds.	MEAN	RANGE OF MEAN $\pm$ 2 S.Ds.
<u>MALES</u>				
21 - 29:	1.50 (154)	1.03 - 1.98	32 μ U/ml	11 - 96 μ U/ml
30 - 39:	1.53 (163)	1.04 - 2.03	34 μ U/ml	11 - 107 μ U/ml
40 - 49:	1.53 (169)	1.06 - 2.00	34 μ U/ml	11 - 100 μ U/ml
50 - 59:	1.54 (142)	1.02 - 2.07	35 μ U/ml	10 - 118 μ U/ml
60 - 69:	1.63 (60)	1.23 - 2.04	43 μ U/ml	17 - 110 μ U/ml
70+ :	1.70 (37)	1.11 - 2.30	50 μ U/ml	13 - 170 μ U/ml
<u>FEMALES</u>				
21 - 29:	1.59 (187)	1.18 - 2.01	39 μ U/ml	15 - 102 μ U/ml
30 - 39:	1.59 (210)	1.12 - 2.07	39 μ U/ml	13 - 118 μ U/ml
40 - 49:	1.61 (168)	1.12 - 2.09	41 μ U/ml	13 - 123 μ U/ml
50 - 59:	1.65 (107)	1.21 - 2.09	45 μ U/ml	16 - 123 μ U/ml
60 - 69:	1.75 (56)	1.32 - 2.18	56 μ U/ml	21 - 151 μ U/ml
70+ :	1.88 (13)	1.47 - 2.29	76 μ U/ml	30 - 195 μ U/ml

(B)

AGE (years)	<u>MEAN "ONE-HOUR" BLOOD- SUGAR (mg/100ml)</u>		<u>MEAN PERCENTAGE DESIRABLE WEIGHT (%)</u>	
	<u>MALES</u>	<u>FEMALES</u>	<u>MALES</u>	<u>FEMALES</u>
21-29	81	79	102	108
30-39	87	85	103	111
40-49	88	93	106	108
50-59	95	94	104	112
60-69	95	104	105	113
70+	113	102	101	111

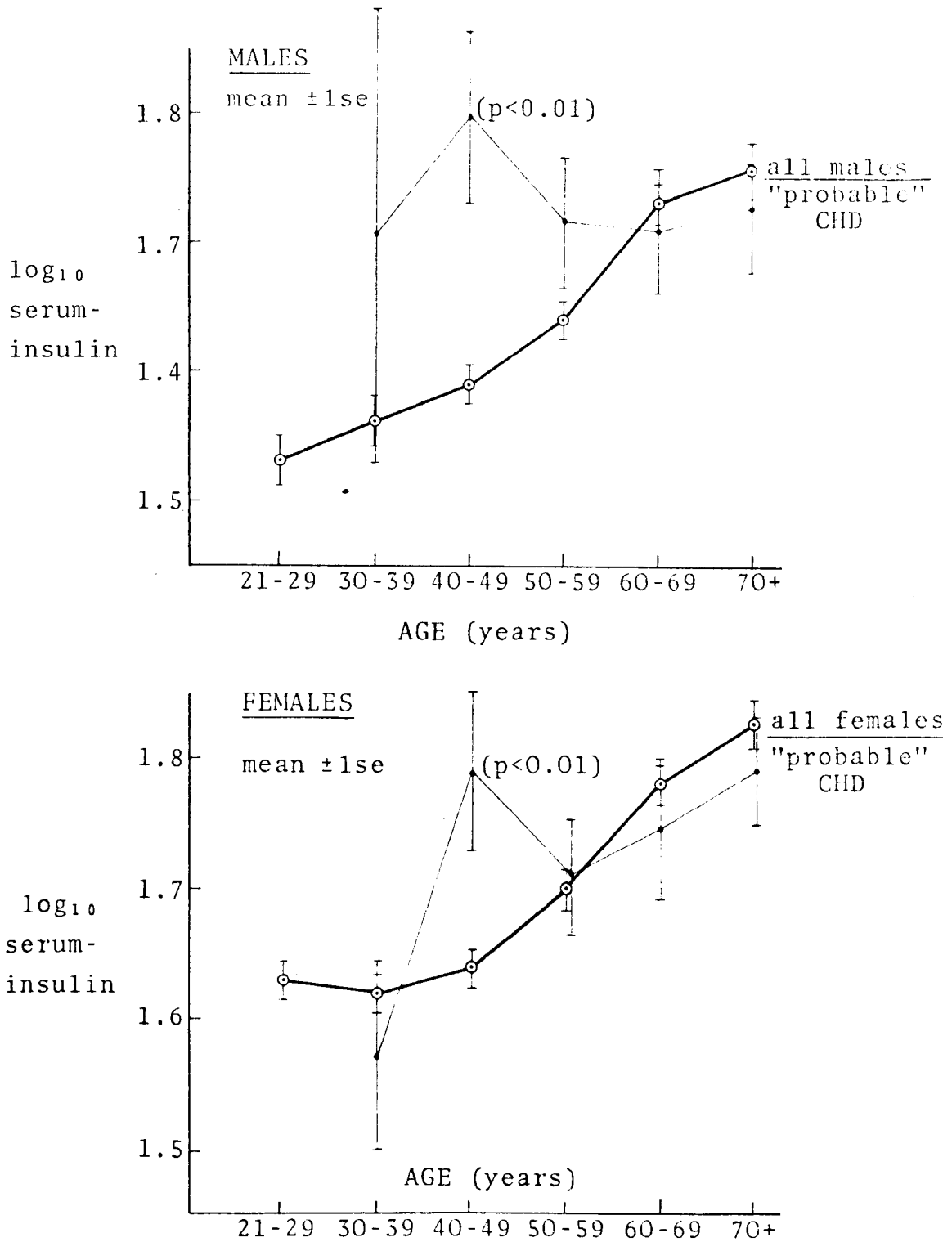


FIGURE 18. Mean "one hour" $\log_{10}$ serum-insulin (± 1 standard error) for subjects with "probable" coronary heart disease.

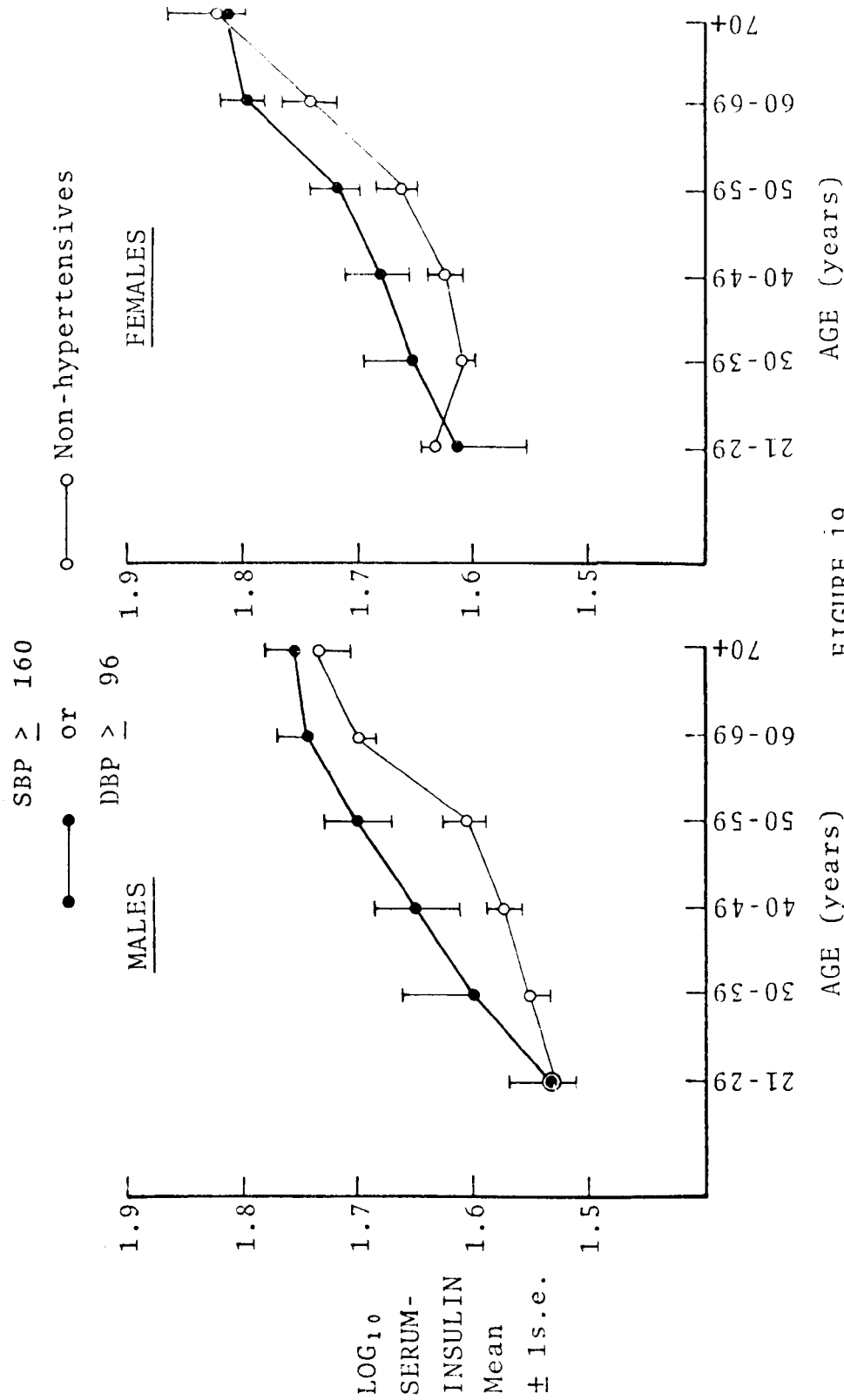


FIGURE 19.

Hypertension and the "one hour" serum-insulin, by age and sex. The mean values $\pm$ 1 standard error for log₁₀ serum-insulin are shown in hypertensive subjects (systolic blood pressure greater than 160mm.Hg. and/or diastolic blood pressure greater than 96mm.Hg.).

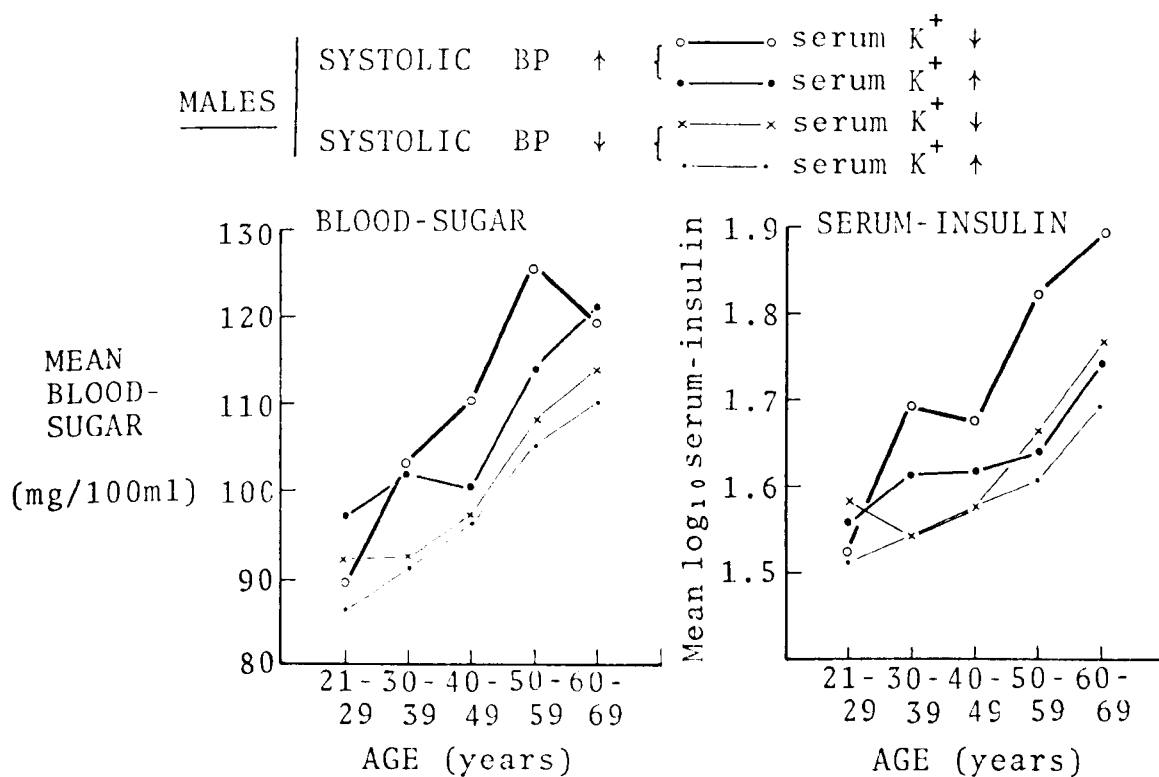


FIGURE 20.

Mean "one hour" blood-sugar levels and log₁₀ serum-insulin for males with "upper 1/5" range versus "lower 4/5" range systolic blood-pressures according to serum potassium in the "lower 1/5" or "upper 4/5" range. The ranges are age- and sex- specific. Hypertensive males with low serum potassium tend to have elevated blood-sugar and serum-insulin levels.

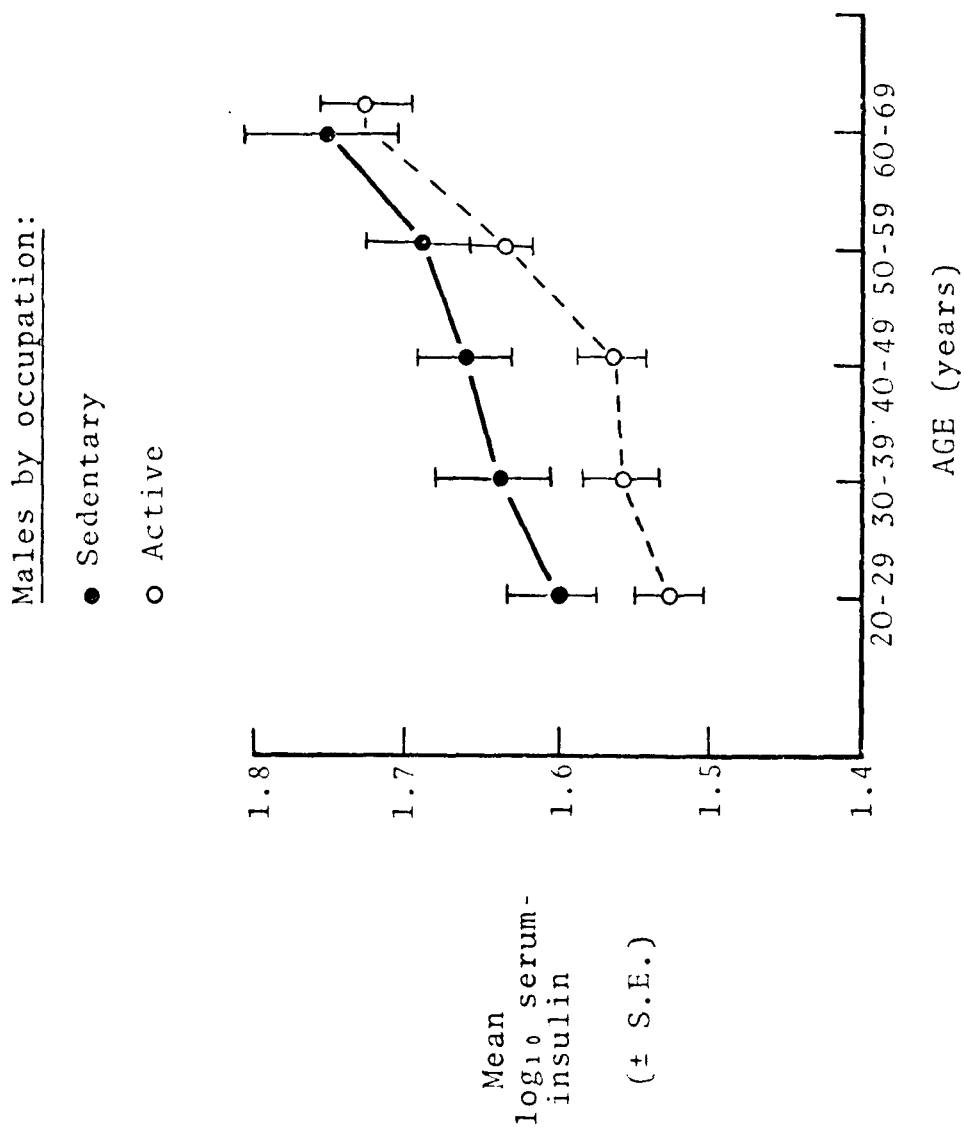


FIGURE 21 Mean $\log_{10}$ serum-insulin ($\pm$ 1 standard error) for males with active, and those with sedentary occupations, by age. (See also Table 30.)

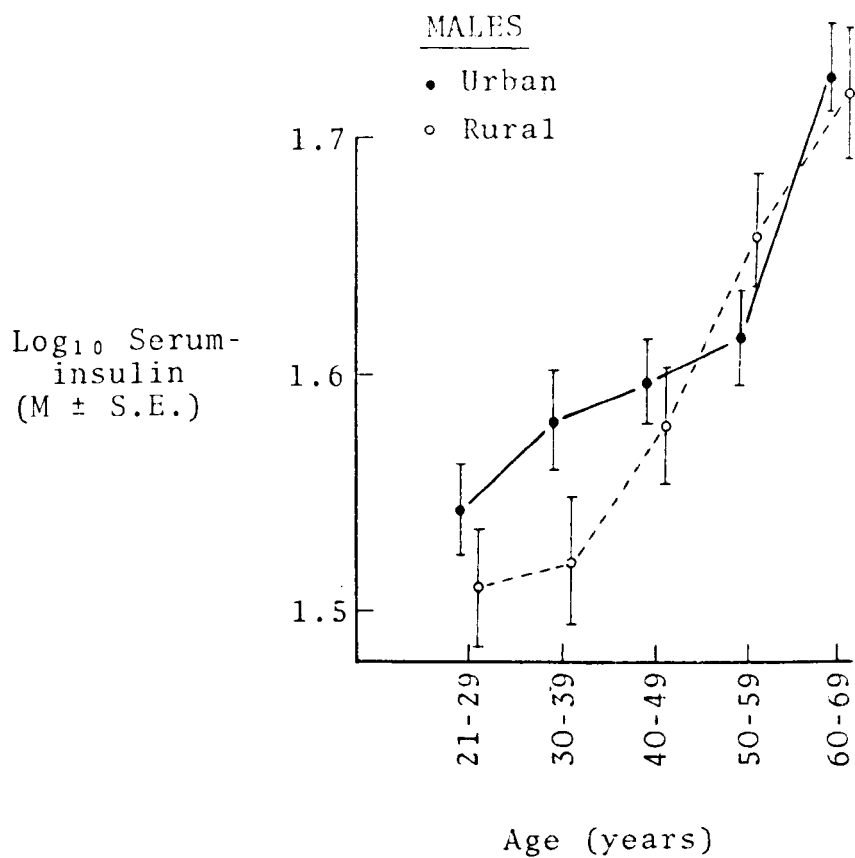


FIGURE 22.

Comparison of the mean "one-hour" log₁₀ serum-insulin for rural versus urban males. The range of ± 1 standard error is shown.

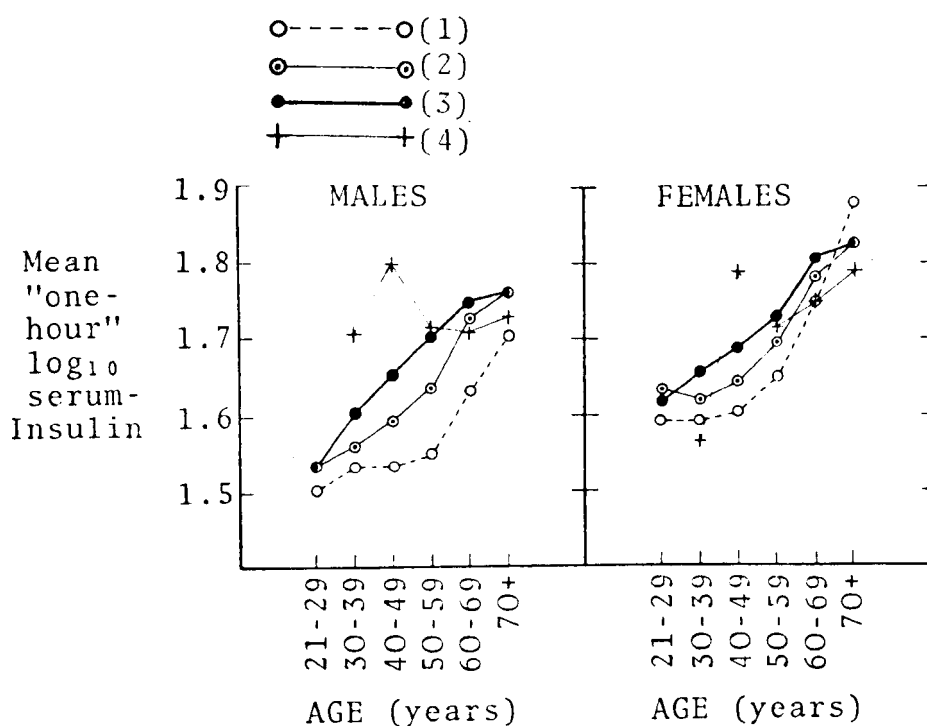


FIGURE 23.

Comparison of the mean "one-hour" $\log_{10}$ serum-insulin values for the groups -

- (1) "Ideal" subjects - obesity, vascular disease hyperglycaemia excluded.
- (2) Total population
- (3) Hypertensive subjects.
- (4) Coronary heart disease subjects.

by 10 year-age groups.

CHAPTER 7

FASTING SERUM-INSULIN LEVELS IN SURVEY SUBJECTS

INTRODUCTION

To study factors related to fasting serum-insulin levels, further blood samples were obtained in a second survey of Busselton subjects in July 1968. The survey was designed to obtain more complete clinical and biochemical documentation of previous respondents with the diagnosis of "suspect" or "probable" coronary heart disease, but with reference to control subjects drawn from the same community. Fasting samples were more desirable than samples from fed subjects for the accurate characterization of blood lipid levels and the serum-insulin levels also would be less subject to variability, although more difficult to measure.

METHODS

POPULATION SAMPLE.

Subjects with a diagnosis of "probable" or "suspect" coronary heart disease (CHD), aged 60 years or less in the 1966 survey, were invited to have further tests for the more complete elucidation of histories of chest pain and/or unusual cardiogram tracing (for diagnostic criteria see Chapter 6, and also Welborn et alii 1969). The overall response rate from 201 CHD subjects was 88%. Arising from the findings of the second survey, 6 males and 5 females, with previously "suspect" CHD diagnoses were transferred to the "probable" CHD category. There were then 44 males, and 32 females with "probable" CHD whose results were analyzed. It was found that

the mean age of the CHD groups (48 years) was a little higher than the control groups (44 years). The remaining subjects with "suspect" CHD have not been included in this report, since their status is undetermined, and the data from this group showed trends intermediate to the control and "probable" groups.

For the sample of "control" subjects, a truly random sampling procedure was adopted with the aim of obtaining acceptances from 5, 10, 20 and 25 males, and the same number of females, in the age ranges 21-29, 30-39, 40-49, and 50-60 years. These numbers were selected in an attempt to provide suitable matching for the age and sex distribution of the CHD subjects. The response rate of these subjects, asked to volunteer for research reasons, was approximately 70%. There were 45 males and 49 females in the control group. Only one known diabetic subject attended and his results were excluded from the analysis.

PLAN OF STUDY.

Over a six-day period in July 1968, subjects were given appointments to attend between 7 a.m. and 11 a.m. It was stressed that nothing other than plain water was to be taken from 10 p.m. of the preceding day, and that exertion (other than dressing and travelling) and smoking were to be avoided. Appointment times were randomized so as to avoid the possible interfering effect of time of day. At the survey centre, history, venous blood sample, physical examination, and electrocardiogram were obtained from each subject.

LABORATORY METHODS.

Blood-sugar levels were measured on cooled heparin-fluoride samples within 36 hours by the Autoanalyzer ferricyanide micro-method (Hoffman 1937) for which the coefficient of

variation of a standard serum in the fasting range was 5%. Serum triglycerides were measured on deep-frozen samples by the method of Lofland (1964), and serum cholesterol and uric acid were measured by Autoanalyzer. All of the above estimations were performed in the Biochemistry Department, Royal Perth Hospital.

Samples for free fatty acid and ketone estimation were snap-frozen and were freighted on dry ice to the University Laboratory of Physiology, Oxford, where serum free fatty acid levels were measured by Duncombe's method (1964) and whole blood ketones by the enzymatic method of Williamson et alii (1962), by courtesy of Dr D.J. Jenkins and Dr J.M. Williamson, respectively.

Assay of serum-insulin levels was performed on deep-frozen samples by the semi-automated double-antibody method and values were computed as described in Chapter 6. The assay conditions were manipulated to provide standard curves sensitive over the range of fasting values anticipated. The modifications involved using half the usual concentrations of guinea-pig anti-insulin serum (1/32,000) and of insulin-I¹²⁵ (1.5 μ U per assay tube) and prolonging incubation of the first antibody reaction to 12 days. The same batch of insulin-I¹²⁵ was used in each of the assays. All samples were assayed in duplicate in 0.1 ml volumes.

Quality control methods for the insulin assay included assessment of standard curves and of standard serum samples. From visual checking of the graphed data, the "sensitivity" of 3 standard curves in each of three assays was $\leq 2.2 \mu\text{U/ml}$. Table 1 shows the mean values obtained by computed curve-fitting for the crystalline insulin solutions of the 9 standard curves. These are close to the expected values, with a mean coefficient of variation of 13%. The mean values of the

assay metameter (percentage insulin-I¹²⁵ precipitated) for the insulin standards is also shown. Standard serum "B" (Chapter 6) was again estimated, both undiluted and diluted 1/10. Table 2 shows that the mean values obtained (32.5 μ U/ml and 3.7 μ U/ml) were consistent with the previous mean value of 34 μ U/ml from 27 assays, with corresponding coefficients of variation of 13.5% and 12.6% respectively. Thus the accuracy of this lower range assay was equivalent to that obtained from the data in the previous chapter.

RESULTS

THE FASTING LEVELS OF SERUM-INSULIN

The fasting levels of insulin were log-normally distributed. Table 3 shows the mean values, with one standard deviation, for log₁₀ serum-insulin of 46 control males and 49 control females, in comparison with 44 males and 32 females with "probable" CHD. Females tended to have higher fasting serum-insulin levels than males, and CHD subjects higher values than controls, but the differences were very small and non-significant. The Table also shows the equivalent values in μ U/ml for the log-mean, and the absolute range encountered, which is ninefold.

No consistent effect of age was discerned, as shown in Table 4, except for the possible tendency, in males and females with CHD, to have higher fasting levels above the age of 44 years, but this was not statistically significant.

CORRELATIONS OF FASTING SERUM-INSULIN WITH OTHER VARIABLES MEASURED.

The mean levels, with one standard deviation, of other variables measured in the 4 groups is shown in Table 5. Males and females with CHD had slightly higher fasting blood-sugar levels, and percentages of ideal weight than the controls, but

a more definite elevation of mean serum triglyceride and possibly of mean serum cholesterol levels was evident. In both sexes, serum free fatty acid levels were higher in CHD subjects, and in CHD females, blood ketones were elevated as compared with control females. Between the CHD and control groups the only difference of statistical significance was that of the serum triglyceride levels in males ($P < 0.05$). The mean time of day of blood sampling was comparable in the 4 groups. (The wider range in control males reflects the fact that 4 subjects attended prior to working hours at 5 a.m.)

The coefficients of correlation of these variables with the fasting $\log_{10}$ serum-insulins are listed in Table 6. Males showed the highest correlation coefficients between insulin and percentage desirable weight ($r = 0.37, 0.65$; $P < 0.001$), and females between insulin and serum triglycerides ($r = 0.65, 0.57$; $P < 0.001$). In all 4 groups, there were consistent positive correlations with serum triglycerides, percentage desirable weight, blood-sugar, and serum uric acid levels, with statistical significances as shown. The remaining associations were not consistent through the groups and were of borderline significance; the positive correlation with serum cholesterol in the control males, and with serum free fatty acids in CHD males; and the negative correlation with blood ketones in CHD females, and with time of day in control females. Significant correlations were also found between the fasting $\log_{10}$ serum-insulin and blood pressure. The correlation coefficients were more strong for diastolic blood pressure - 0.31 ($P < 0.05$) and 0.44 ($P < 0.01$) for male and female controls; and - 0.32 ($P < 0.05$) and 0.32 (not significant) for male and female CHD subjects, respectively.

The inter-relationship between certain of these variables are demonstrated in the correlation matrix for males in Table 7, and for females in Table 8. These show the general tendency for the $\log_{10}$ serum-insulin values to have correlations with serum triglycerides, percentage desirable weight, and blood-sugar of equal or greater magnitude than the correlations of these with the other variables. (It is pertinent to mention that serum free fatty acids showed no significant correlation with serum triglycerides - the correlation coefficients ranging from -0.20 to + 0.23.)

MULTIPLE REGRESSION ANALYSIS.

With $\log_{10}$ serum-insulin as the dependent variable, and as determining variables those listed in Table 5, multiple regression equations were computed for each of the 4 groups, and the results are shown in Tables 9 and 10. Because of the comparatively small numbers, no attempt was made to derive other than linear associations. Summarizing the results, serum triglyceride levels were found to be significant independent "determinants" of the fasting $\log_{10}$ serum-insulin in all 4 groups of subjects; and percentage desirable weight also showed significant associations with insulin levels in 3 groups (the exception being females with CHD). Percentage desirable weight was the more powerful "predictor" for males ($P < 0.001$) but serum triglyceride showed the stronger association with insulin in females ($P < 0.001$). The nature of these independent associations, as calculated from the multiple regression equations, is depicted in Figure 1. The regression lines of serum triglyceride on $\log_{10}$ serum-insulin correspond closely in control as compared to CHD subjects: no essential difference in the nature of the relationship can be discerned. CHD subjects do not appear to have any abnormal tendency to show higher triglyceride

levels for a given insulin level. The independent relationships of percentage desirable weight and fasting serum-insulin were very similar in control and CHD males, but the effect of obesity in males was more marked than in females (Figure 1) - a trend that was also seen with the "one hour" serum-insulin (Chapter 6).

The remaining associations of significance in the 4 equations (Table 9) are interpreted with caution since none are consistent through the groups, and the numbers of subjects are small. Time of day has a small negative influence in the control males. In CHD males the negative association of blood ketones, and the positive association of serum free fatty acids are difficult to interpret since these two variables were strongly inter-correlated.

The multiple correlation coefficients (R) are high (0.57 to 0.77) and the "unbiased" indices of determination (A) indicate that about half of the total variability of the fasting serum insulin levels could be ascribed to the independent contribution of these associated factors.

BIOCHEMICAL MEASUREMENTS TENDING TO DISCRIMINATE THE "CHD" SUBJECTS.

Although the mean levels of fasting $\log_{10}$ serum-insulin were only slightly elevated in the CHD groups, the distributions of values were such that probably greater than the expected 20% of subjects with CHD had insulin values exceeding the 80th percentile levels of the corresponding control males and females. This is evident from Table 11, which also shows similar data for serum cholesterol, triglycerides, and blood-sugar. Only the differences for serum triglyceride in males ($P < 0.05$) and females ($P < 0.05$), and for elevated serum-insulin in females ($P < 0.05$) were significant by the chi-squared test.

DISCUSSION

To do this study, particular care had first to be taken to obtain accurate measurements of the low levels of insulin found in the fasting state, by manipulating the concentrations of assay reactants and by prolonging the incubation time to achieve equilibrium conditions. The quality control data provide assurance that the level of performance of these more sensitive assays was equivalent to that of the usual procedure, and in addition the values observed for the serum standards were consistent with those of the previous assay series.

The mean insulin levels obtained for the control subjects (10-12 μ U/ml) corresponded to the mean fasting levels (12 μ U/ml) reported by Chlouverakis et alii (1967) from a normoglycaemic sample of the Bedford population. Various other workers have found somewhat higher mean values in fasting healthy subjects, of the order of 20 μ U/ml (see Table 12, Chapter 3) but their reports have indicated no special care in the measurement of fasting levels and it is possible that assay artefacts contributed to the higher readings. Between the sexes, and between control and coronary heart disease groups, the fasting serum-insulin levels showed no significant difference, and age had no discernable effect.

However, significant positive correlations were found between fasting $\log_{10}$ serum-insulin and the blood-sugar levels: which suggests that degrees of insulin resistance were discernable, even over the narrow range of basal values. Serum-insulin was correlated most strongly with percentage desirable weight and with serum triglyceride levels; and further multiple regression analysis established the independent association of these two variables with $\log_{10}$ serum-insulin. When these were taken into account, blood-sugar levels had no independent relationship with the serum-insulin levels.

An important finding of this study was the strong positive association of the fasting serum-insulin levels with the triglyceride levels, demonstrated in a sample of the general population as well as in subjects with coronary heart disease. This consistent relationship existed in all of the groups studied, and was shown to be independent of other factors including the blood-sugar and free fatty acid levels, and the degree of obesity. Previous reports refer predominantly to patients with premature atherosclerosis or hyperlipidaemia. Such patients, in response to a high carbohydrate diet, produced a "steady state" of elevated triglyceride levels that correlated closely with the actual insulin response to the diet as well as correlating with glucose tolerance (Farquhar et alii 1966). Obesity and hypertriglyceridaemia alone were unlikely to have contributed to the metabolic response, since subjects with these conditions, but with low insulin levels, failed to develop hypertriglyceridaemia (Reaven et alii 1967). Apart from the present study, the only other normal sample investigated showed a very strong correlation between a mean glucose tolerance index of blood-sugar levels and the fasting serum triglyceride level, but a less powerful association existed between the latter and the mean serum-insulin response. Finally, with respect to the positive inter-relations of serum-insulin, blood-sugar, and triglyceride levels, it is worth mentioning that a single case of insulinoma, with high insulin but low blood-sugar levels, did not exhibit hypertriglyceridaemia (Farquhar et alii 1966).

All of these observations suggest that a direct physiological relationship exists between insulin and triglyceride levels, in the presence of normal or elevated blood-sugar levels. They are compatible with the hypothesis that insulin promotes hepatic triglyceride secretion. The liver is presumed to be the major source of endogenous circulating

triglycerides, but this is difficult to prove. Doubt also exists as to the main substrates for hepatic triglyceride, for although circulating free fatty acids were initially proposed for this role (Havel 1961; Nestel 1967) the possibility that glucose could contribute to the fatty acid moiety of hepatic triglyceride was not initially considered. The clinical studies reviewed above suggest that although insulin shows the major association with triglyceride levels, blood-sugar levels also contribute. In the present investigation, and in the report of Reaven et alii (1967), free fatty acid levels and triglyceride levels were not correlated. In support of the role of insulin plus glucose in promoting hepatic triglyceride synthesis, "in vitro" studies of rat liver slices have demonstrated the production of triglyceride-fatty acid from labelled glucose, a phenomenon that was considerably augmented by insulin pre-treatment of the animals (Salans and Reaven 1966).

It is not possible to say from the available information whether the fasting serum-insulin directly relates to the triglyceride level, or whether the latter reflects repeated post-prandial elevations of circulating insulin. The distinction is, perhaps, a fine one because high fasting levels of insulin, or high post-prandial levels, both imply "insulin resistance". Thus insulin resistance (whatever the cause) may underlie "insulin-induced hypertriglyceridaemia". In this situation, the liver has been proposed as the passive victim of elevated insulin levels, together with normal or elevated blood-sugar levels (Reaven 1967). Increased dietary carbohydrate would tend to accelerate triglyceride production particularly in insulin resistant subjects. In fact, insulin resistance has been positively identified in hypertriglyceridaemic subjects by use of insulin tolerance tests (Davidson and Albrink 1965) and by glucose tolerance tests in which high levels of endogenous insulin have been found in the presence of normal blood-sugar levels (Davidson and Albrink 1966).

The positive association between fasting insulin levels and triglyceride levels probably does not reflect any "peripheral" interaction of the two; partly because in peripheral tissues insulin appears to promote the hydrolysis of glycerides by increasing lipoprotein lipase activity (Kessler 1962) and if this were a major action a negative relationship should become apparent. The possibility that elevated triglyceride levels themselves contribute to insulin resistance perhaps warrants study.

The failure to demonstrate any substantial increase in fasting insulin levels of coronary heart disease subjects has two possible explanations. Subjects with coronary heart disease may exhibit exaggerated insulin responses only after glucose loading, but may have fasting levels of insulin comparable with control subjects, as found in the studies of Nikkila et alii (1965) and Tzagournis et alii (1967). If fasting serum-insulin levels are elevated in true coronary heart disease cases (Peters and Hales 1965; Christiansen et alii 1968), the presence of undetected atheroma in the control subjects and the undetermined effects of treatment of disease cases, may have obscured real differences. It must be pointed out, however, that the coronary heart disease subjects exhibited the usual tendency to have elevated cholesterol and triglyceride levels, and tended to show greater than the expected proportion of subjects with fasting serum-insulin levels above the 80th percentile value of the controls.

Serum triglyceride levels may prove to be of value in discriminating the risk of coronary heart disease (Carlson 1960; Albrink et alii 1961; Canellos and Hatch 1962) although definitive conclusions are required from a prospective comparison of control and disease subjects drawn from the same population. If, as seems likely, serum triglyceride levels enables the "prediction" of cardiovascular disease, the

association of insulin and triglyceride levels assumes further importance. High insulin levels determine hypertriglyceridaemia: evidence has been reviewed (Chapter 6) that high insulin levels are implicated in the genesis of atherosclerotic lesions.

SUMMARY

Fasting blood-samples were obtained from control subjects (46 males, 49 females) and from subjects with a diagnosis of "probable" coronary heart disease (44 males, 32 females) under the age of 60 years, as a follow-up study of the Busselton community. The mean fasting levels of control subjects were close to 10 μ U/ml and no significant effect of age, sex or of coronary heart disease were found. Fasting serum-insulin and triglyceride levels showed consistently significant independent associations, similar in control and coronary heart disease subjects, that probably reflect a physiological role of insulin in promoting hepatic triglyceride synthesis. Hypertriglyceridaemia may provide additional evidence of "insulin resistance". Fasting serum-insulin levels were also correlated with fasting blood-sugar and serum uric acid levels, and with percentage desirable weight, but only the latter was independently associated with insulin values, and its effect was more marked in males than in females. No consistent correlations were found between fasting serum-insulin and free fatty acid or blood ketone levels. Epidemiological proof of the association between hypertriglyceridaemia and cardiovascular disease will provide further support for insulin as an aetiological factor in atherosclerosis.

TABLE 1. SUMMARY OF ABSOLUTE VALUES OBTAINED FOR CRYSTALLINE INSULIN STANDARDS BY THE COMPUTED CURVE-FITTING PROCEDURE IN 3 ASSAYS (ADJUSTED TO BE SENSITIVE TO FASTING LEVELS OF INSULIN).

CONCENTRATION OF INSULIN STANDARDS ($\mu\text{U/ml}$)					MEAN ASSAY METAMETER
EXPECTED VALUES	COMPUTED VALUES				% Insulin-I ¹²⁵ bound
	Mean	$\pm$	S.D.	(V%)	
2.2	2.4	$\pm$	0.4	(15%)	37.1%
4.4	4.2	$\pm$	0.5	(12%)	35.6%
8.8	8.9	$\pm$	1.1	(12%)	30.0%
17.6	18.6	$\pm$	1.3	(7%)	24.4%
35.3	36.2	$\pm$	3.7	(10%)	18.5%
70.5	75.1	$\pm$	15.0	(20%)	14.2%
141.0	146.2	$\pm$	26.4	(18%)	11.1%

S.D. = Standard deviation

V% = Coefficient of variation

TABLE 2. Insulin values obtained for standard serum "B" (undiluted and diluted 1/10) through three assays. The coefficient of variation are given.

ASSAY NUMBER	INSULIN VALUES (μ U/ml) FOR SERUM STANDARDS -	
	"B"	"B X 1/10"
1	29	3.9
"	29	3.6
"	30	3.5

2	42	3.2
"	35	3.0
"	30	3.8

3	35	4.4
"	34	4.3
"	29	3.9
MEAN	32.5 μ U/ml	3.73 μ U/ml
S.D.	4.4 "	0.47 "
Coefficient of variation	13.5%	12.6%

TABLE 3. Fasting serum-insulin levels obtained in "control" males and females, and those with a survey diagnosis of "probable" coronary heart disease. The mean $\log_{10}$ serum-insulin levels, with one standard deviation (S.D.) are given, and the corresponding log-means and absolute ranges (in U/ml).

FASTING SERUM-INSULIN		
SUBJECTS (N = number)	MEAN $\log_{10}$ VALUES $\pm$ S.D.	LOG MEAN (μ U/ml) AND RANGE
Control males (N = 46)	1.019 $\pm$ 0.187	10 (5-45) μ U/ml
"CHD" males (N = 44)	1.071 $\pm$ 0.174	12 (5-28) μ U/ml
Control females (N=49)	1.073 $\pm$ 0.163	12 (6-46) μ U/ml
"CHD" females (N=32)	1.121 $\pm$ 0.183	13 (5-27) μ U/ml

TABLE 4. Fasting $\log_{10}$ serum-insulin in "control" and coronary heart disease subjects, by age groups.

FASTING LOG_{10} SERUM-INSULIN				
AGE (years)	MALES		FEMALES	
	Control	CHD	Control	CHD
20 - 39:	1.06 (13)	0.98 (8)	1.11 (13)	1.06 (7)
40 - 49:	0.97 (12)	1.10 (10)	1.02 (18)	1.13 (5)
50 - 60:	1.02 (21)	1.09 (26)	1.10 (18)	1.14 (20)

VARIABLE (and units)	MALES		FEMALES	
	Control (N = 46)	CHD (N = 44)	Control (N = 49)	CHD (N = 32)
Age (years)	44 ± 10	48 ± 10	44 ± 11	48 ± 10
Blood sugar (mg/100ml)	84 ± 9	89 ± 10	84 ± 11	87 ± 13
Percent. ideal weight	110 ± 16	114 ± 16	120 ± 24	124 ± 23
S. Triglycerides (mg/100 ml)	127 ± 31	159 ± 72	119 ± 40	145 ± 48
S. Cholesterol (mg/100ml)	237 ± 38	250 ± 53	231 ± 42	249 ± 56
S. Uric acid (mg/100 ml)	5.4 ± 1.0	5.9 ± 1.5	4.3 ± 1.0	4.7 ± 1.3
S. Free fatty acid (mg/100 ml)	363 ± 127	397 ± 166	460 ± 181	522 ± 210
Plasma ketones (mg/100 ml)	47 ± 27	48 ± 33	56 ± 36	77 ± 66
Time of day (hours)	9.2 ± 1.6	9.2 ± 1.1	9.2 ± 0.9	9.4 ± 1.0

TABLE 5. Variables measured in fasting "control" subjects.
Mean values ± one standard deviation are shown.

COEFFICIENTS OF CORRELATION OF FASTING $\log_{10}$ SERUM-INSULIN

VARIABLE	MALES		FEMALES	
	Controls (N = 46)	CHS (N = 44)	Controls (N = 49)	CHD (N = 32)
Percentage ideal weight	0.57 (p< 0.001)	0.65 (p< 0.001)	0.51 (p<0.001)	0.30 (N.S.)
Serum triglycerides:	0.37 (p< 0.05)	0.48 (p< 0.01)	0.65 (p<0.001)	0.57 (p<0.001)
Blood sugar:	0.30 (p< 0.05)	0.43 (p< 0.01)	0.53 (p<0.001)	0.25 (N.S.)
Serum uric acid:	0.10 (N.S.)	0.39 (p< 0.01)	0.29 (p<0.05)	0.28 (N.S.)
Serum cholesterol:	0.35 (p< 0.05)	0.05 (N.S.)	0.07 (N.S.)	0.04 (N.S.)
Serum free fatty acid:	-0.02 (N.S.)	0.30 (p< 0.05)	0.09 (N.S.)	-0.23 (N.S.)
Plasma ketones:	-0.17 (N.S.)	0.03 (N.S.)	0.06 (N.S.)	-0.41 (p<0.05)
Age:	-0.03 (N.S.)	0.20 (N.S.)	0.04 (N.S.)	0.26 (N.S.)
Time of day:	-0.25 (N.S.)	0.17 (N.S.)	-0.32 (p<0.05)	-0.14 (N.S.)

TABLE 6. Variables related to the fasting $\log_{10}$ serum-insulin values in control subjects and those with coronary heart disease (CHD). Simple correlation coefficients are shown for each variable with $\log_{10}$ serum-insulin, and their significance. (Units for the variables listed are as shown in Table 5.)

TABLE 7. COEFFICIENTS OF CORRELATION FOR MALES BETWEEN THE FASTING LOG_{10} SERUM-INSULIN AND RELATED VARIABLES. ON THE RIGHT OF THE DIAGONAL, THE CORRELATION COEFFICIENTS ARE GIVEN FOR CONTROL MALES (ABOVE) AND FOR CHD MALES (BELOW). CORRESPONDING SIGNIFICANCE LEVELS ARE GIVEN TO THE LEFT OF THE DIAGONAL.

LINEAR CORRELATION COEFFICIENTS						
Above: Control males			Below: CHD males			
	<u>Log_{10} insulin</u>	<u>Tri- glyc.</u>	<u>% wt.</u>	<u>Uric acid</u>	<u>Blood sugar</u>	<u>Chol.</u>
Log_{10} insulin:		.37 .48	.57 .65	.10 .39	.30 .43	.35 .05
S. triglyceride:	* **		.12 .44	.01 .43	-.07 .36	.39 .35
% ideal weight:	*** ***	- **		.05 .54	.38 .25	.20 .18
S. uric acid:	- **	- ***	- ***		-.18 .23	-.15 -.12
Blood-sugar:	* **	- *	** -	- -		.24 .18
S. cholesterol:	* -	** *	- -	- -	- -	

- Not significant

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

TABLE 8. COEFFICIENTS OF CORRELATION FOR FEMALES BETWEEN THE FASTING LOG_{10} SERUM-INSULIN AND RELATED VARIABLES. ON THE RIGHT OF THE DIAGONAL, THE CORRELATION COEFFICIENTS ARE GIVEN FOR CONTROL FEMALES (ABOVE) AND FOR CHD FEMALES (BELOW). CORRESPONDING SIGNIFICANCE LEVELS ARE GIVEN TO THE LEFT OF THE DIAGONAL.

LINEAR CORRELATION COEFFICIENTS						
Above: Control females			Below: CHD females			
	<u>Log_{10} insulin</u>	<u>Tri- glyc.</u>	<u>% wt.</u>	<u>Uric acid</u>	<u>Blood sugar</u>	<u>Chol.</u>
Log_{10} insulin:		.65 .57	.51 .30	.29 .28	.53 .25	.07 .04
S. triglyceride:	*** ***		.39 .25	.38 .42	.63 .36	.31 .26
% ideal weight:	*** -	** -		.43 .52	.26 .38	.19 .09
S. uric acid:	* -	** -	** **		.19 .58	.39 .00
Blood-sugar:	*** -	*** *	- *	- ***		-.07 .03
S. cholesterol:	- -	* -	- -	** -	- -	

- Not significant

* $P < 0.05$

** $P < 0.01$

*** $P < 0.01$

MULTIPLE REGRESSION ANALYSIS OF THE FASTING LOG₁₀ SERUM-INSULIN

FACTOR	CONTROL MALES (N = 46)		CHD MALES (N = 44)	
	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE
S. triglycerides	+ 0.171 x 10 ⁻²	2.5 (p<0.05)	+ 0.104 x 10 ⁻²	3.3 (p<0.01)
% ideal weight	+ 0.626 x 10 ⁻²	4.8 (p<0.001)	+ 0.525 x 10 ⁻²	4.0 (p<0.001)
Time of day	- 0.292 x 10 ⁻³	2.3 (p<0.05)	...	...
Plasma ketones	...	...	- 0.230 x 10 ⁻²	3.2 (p<0.01)
S. free fatty acids	...	...	+ 0.314 x 10 ⁻³	2.5 (p<0.05)
Y INTERCEPT	+ 0.379		+ 0.290	

MULTIPLE CORRELATION COEFFICIENT (R)

0.691 0.771

PROPORTION OF VARIABILITY ASCRIBABLE TO REGRESSION ("A")

0.441 0.553

TABLE 9

RESULTS OF MULTIPLE REGRESSION ANALYSIS OF FACTORS DETERMINING THE FASTING LOG₁₀ SERUM-INSULIN IN 46 CONTROL MALES AND 44 MALES WITH CORONARY HEART DISEASE (CHD). THE UNITS FOR THE STATED FACTORS ARE AS SHOWN IN TABLE 5. THE STUDENT'S "T" VALUE INDICATES THE SIGNIFICANCE OF EACH REGRESSION COEFFICIENT.

MULTIPLE REGRESSION ANALYSIS OF THE FASTING LOG₁₀ SERUM-INSULIN

FACTOR	CONTROL FEMALES (N = 49)		CHD FEMALES (N = 32)	
	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE	REGRESSION COEFFICIENT	STUDENT'S "T" VALUE
S. triglycerides	+ 0.216 x 10 ⁻²	4.7 (p<0.001)	+ 0.219 x 10 ⁻²	3.8 (p<0.001)
% ideal weight	+ 0.212 x 10 ⁻²	2.7 (p<0.05)	...	...
Y INTERCEPT	+ 0.561		+ 0.805	

MULTIPLE CORRELATION
COEFFICIENT (R)

0.709 0.574

PROPORTION OF VARIABILITY
ASCRIPTABLE TO REGRESSION ("A")

0.441 0.307

TABLE 10. RESULTS OF MULTIPLE REGRESSION ANALYSIS OF FACTORS DETERMINING THE FASTING LOG₁₀ SERUM-INSULIN IN 49 CONTROL FEMALES AND 32 FEMALES WITH CORONARY HEART DISEASE (CHD)

TABLE 11. DISCRIMINATING VALUE OF BIOCHEMICAL FACTORS:
 THE NUMBER OF CHD SUBJECTS WITH VALUES ABOVE
 THE SEX-SPECIFIC 80TH PERCENTILE LEVELS OF
 THE CONTROL SUBJECTS. PERCENTAGES ARE SHOWN
 IN PARENTHESES ("EXPECTED" PERCENTAGE FOR
 CONTROL SUBJECTS = 20%).

VARIABLE	NO. OF CHD SUBJECTS HAVING VALUES ABOVE 80TH PERCENTILE VALUE OF CONTROL GROUP			
	CHD males (N=44)		CHD females (N=32)	
S. cholesterol	16	(36%)	12	(37%)
S. triglyceride	19	(43%)	15	(47%)
Blood-sugar	15	(34%)	10	(31%)
Serum-insulin	15	(34%)	14	(44%)

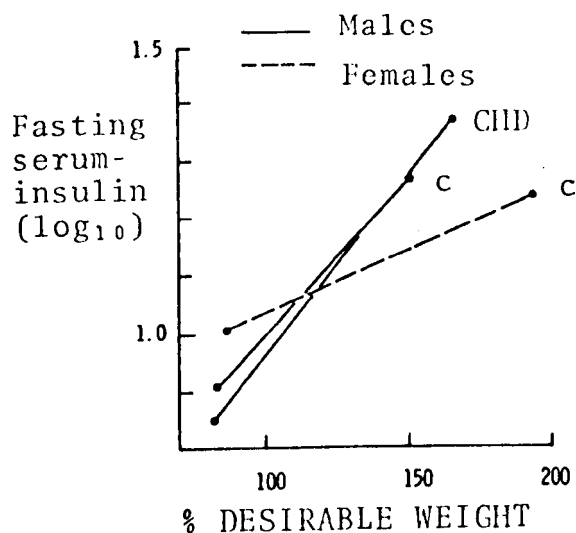
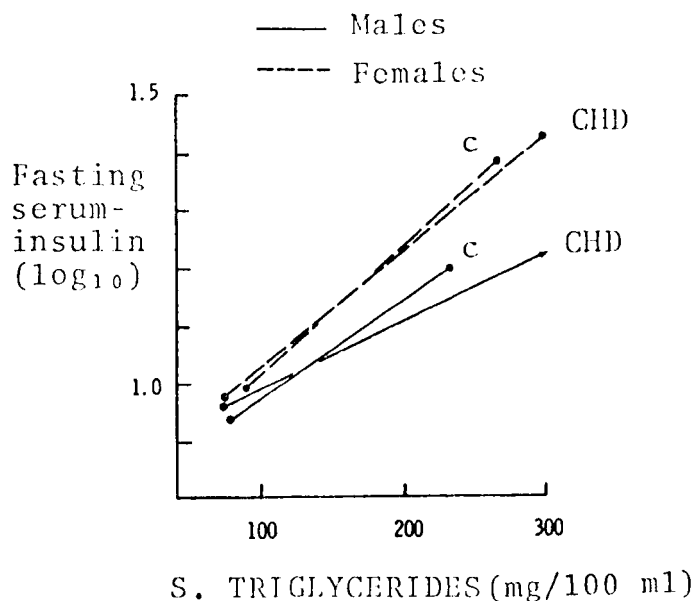


FIGURE 1 The independent association of fasting serum-insulin levels with (a) serum triglycerides; (b) percentage desirable weight; from multiple regression analysis (Tables 9 and 10).

CHD = coronary heart disease subjects
c = control subjects

CHAPTER 8

GENERAL SUMMARY AND CONCLUSIONS

THE INSULIN IMMUNOASSAY.

The double antibody method for the assay of serum-insulin has proved to be a robust technique in that it has shown a consistently reliable performance over a long period of time. Modifications directed at achieving complete separation of free from antibody-bound insulin in the presence of the interfering effects of serum were essential for the confident use of the assay. Factors impairing complete precipitation of antibody-bound insulin were identified by using a labelled pure guinea-pig gamma globulin to monitor the reaction. These factors were corrected. The long-term accuracy of assay performance was assessed by repetitive measurements of standard sera; coefficients of variation of 10-14% were consistently achieved. This degree of variability compared well with values reported by other workers. Use of two serum standards has shown a regular parallel drift that indicated unidentified systematic phenomena affecting the assay. Variable integrity of reagents, such as the labelled insulin or the insulin anti-serum, may be responsible for this.

Areas for future improvement of the measurement of insulin include identifying and minimizing sources of variability. In addition, the elimination of cross-reaction of insulin antisera with proinsulin or other inactive precursors may refine the assay's specificity. At present, the extent to which proinsulin interferes with true insulin measurement is undetermined, but appears to be small. Finally, there is a need for further simplification of the

assay technique. The separation stage of free from antibody-bound hormone requires extensive manual processing, and also the maintenance of a batch of laboratory animals to provide immunological precipitating reagents. More recently introduced methods achieve greater simplicity, but at the expense of greater uncertainty about the efficiency of the separation procedure, particularly in the presence of serum proteins. Such methods require careful assessment of their long-term performance as compared with the more established procedures of proven reliability such as chromato-electrophoresis or the double antibody technique. To facilitate these comparisons between laboratories, the provision of standard reference sera as universal quality control material would be invaluable.

THE VARIABILITY OF SERUM-INSULIN LEVELS.

Clinical and epidemiological studies were pursued to identify factors related to the wide range of insulin levels encountered in man. Insulin levels were found to be unimodal, with a pronounced positive skew, but logarithmic transformation of the values resulted in a symmetrical and normal distribution. Use of the logarithmic insulin values is recommended to yield mean levels and statistical ranges that more accurately characterize the data, and in addition, that provide a more valid basis for statistical comparisons.

In a group of lean healthy volunteers, insulin levels were shown to have a close positive association with the corresponding blood-sugar levels. This relationship existed at any given sampling time during the glucose tolerance test, and while it supported the "biological validity" of the insulin measurement, the finding also pointed to an obscure "insulin resistance", since subjects showing consistently higher values for circulating insulin also had higher glucose levels. At

one hour after glucose, the high insulin secretors showed a delayed peak level of insulin, but in the whole group, the positive association between blood-sugar and serum-insulin was the most powerful. The one hour sampling time therefore, which gives a good reflection of overall glucose tolerance, also identifies more closely the degree of "insulin resistance" encountered in normal subjects.

Patients with essential hypertension or peripheral vascular disease but with normal glucose tolerance exhibited substantially elevated mean serum-insulin levels and an increased tendency to show a delayed peak response at one hour. In this vascular disease group, insulin levels rose with increasing age. Diabetic hypertensive subjects, treated with long-term benzothiadiazines, exhibited a spectrum of insulin levels similar to that of obese diabetics, with excessive values in borderline or mild diabetes, but delayed and sub-normal responses with more severe diabetes. In most cases, cessation of benzothiadiazines did not influence glucose tolerance or insulin response, and it is suggested that the diabetes related more to the vascular disease than to the drug therapy. "Insulin resistance" was thus identified in vascular disease, but frank diabetes was associated with impaired secretion of insulin.

In severe obesity, high insulin levels and "insulin resistance" were confirmed in normoglycaemic patients; and in the mild or moderate obese diabetics insulin lack, relative to the non-diabetic obese, was demonstrated. After a 7 day fast, the previously non-diabetic subjects showed impaired insulin secretion, but no good evidence for increased insulin resistance was found by insulin tolerance tests. The uniform elevation of free fatty acid levels therefore did not obviously impair insulin action and the latter mechanism was unlikely to account for "starvation diabetes". The diabetic obese, after a 7 day fast, had improved glucose tolerances with

appropriate insulin responses relative to the blood-sugar levels. After starvation, when basal blood-sugar levels of the groups were comparable, the diabetic obese showed no greater "insulin resistance" than those subjects with simple obesity. Thus insulin resistance is indeed related to obesity, but clinical diabetes (or starvation diabetes) in obese subjects is associated with deficient insulin secretion rather than any further impairment of the action of circulating insulin.

A review of the insulin levels of diabetics, including the obese, those with vascular disease, and others, indicated that with the 50 g. oral glucose tolerance test, the one hour sampling time identified most clearly the delayed and relatively low insulin levels of more severe hyperglycaemia. With blood-sugar levels exceeding 180-200 mg/100 ml, a general trend to show decreasing insulin values occurred and was proportional to the height of the blood-sugar.

A population study was planned to identify, in suitable numbers of subjects, some of the factors related to the wide range of insulin levels encountered in health and disease. Physical and biochemical measurements, including serum-insulin, were obtained in 3,313 adults of the rural township of Busselton (Western Australia), representing 88% of the entire target population. Multiple regression analysis enabled the definition of the independent association of various factors with serum-insulin. Females had higher insulin levels than males, and although the difference was most marked for females of reproductive age, it persisted in the older groups, and may be related, in part, to their increased adiposity. The "one hour" serum-insulin rose progressively with increasing age, and no evidence existed for any decline of insulin secretory capacity in the aged. At 50 years and over the effect of age was independent of the rising blood-sugar and other factors. The strong association of obesity with serum-

insulin was confirmed, and was more marked in males than females. The independent relationship between serum-insulin and relative body weight was linear: thus in the population generally, even the extremes of obesity did not appear to exhaust the secretory reserve of the pancreas.

The blood-sugar level, at one hour after glucose proved to be the major determinant of serum-insulin by multiple regression analysis. A steep positive relationship existed between these two variables with blood-sugars ranging from 30 to 180 mg/100 ml. This again indicated that "insulin resistance" was a major cause of the variability of insulin levels. Above the threshold blood-sugar level of 180 mg/100 ml, serum-insulin levels showed a negative trend with increasing blood-sugar, confirming the impression from clinical studies that, independent of other factors, the severity of diabetes was proportional to the deficiency of circulating insulin. In summary, a general rule may be stated: "borderline" hyperglycaemia as indicated by a "one hour" blood-sugar level of 180 mg/100 ml or less, was determined essentially by factors relating to "insulin resistance"; whereas frank hyperglycaemia, with one hour levels of blood-sugar exceeding 180 mg/100 ml, was related more to insulin deficiency. The lack of insulin probably reflected the "biochemical inertia" of the secretory mechanism.

Of other physiological variables found to have significant independent associations with the "one hour" serum-insulin, the negative relationship of serum potassium concentrations may have reflected a known effect of insulin action, although in hypertensive males suggestive evidence implicated hypokalaemia as a possible cause of insulin resistance. The positive correlation found between serum uric acid levels and serum-insulin is unexplained but may have been mediated by their mutual association with vascular disease, or with lipid metabolites.

A number of factors relating to the test conditions contributed to the variability of insulin levels. The sampling time in relation to the glucose load was clearly important. (Although this is conventionally standardized, at least as useful an indication of the "expected" range of serum-insulin in healthy subjects may be obtained by relating the $\log_{10}$ serum-insulin values to the post-glucose blood-sugar levels). A further test factor with marked influence on the insulin response to oral glucose was shown to be the interval from previous food ingestion. The "food interval" accounted entirely for the circadian variation in insulin responses observed in the population. The insulin response to glucose when administered within 2-3 hours of food was augmented, probably by gastrointestinal factors, although blood-sugar levels were not appreciably influenced. A final test factor of importance is the size of the glucose load administered. In the studies described this was a constant dose (50 g.), but the resulting insulin levels produced were inversely related to the height of individuals. Therefore, for standardized comparisons, the glucose load ideally should be related to height or to a similar index of lean body mass.

The fasting serum-insulin levels, in a follow-up study of Busselton subjects, were found to show a strong association with serum-triglyceride levels in healthy controls as well as in coronary heart disease. This finding is compatible with the hypothesis that insulin has a physiological role in promoting hepatic triglyceride secretion, in the presence of normal or elevated blood-sugar levels.

INSULIN AND DIABETES

The problem of "insulin resistance" is undoubtedly of importance in the genesis of maturity-onset diabetes. The coexistence of high insulin levels with normal or moderately elevated blood-sugar levels has been demonstrated repeatedly in normoglycaemic subjects, and in those with

"borderline" hyperglycaemia. Some independent factors contributing to insulin resistance have been identified, and these include age, obesity, and vascular disease. In search of a unitary hypothesis, it is proposed that impaired vascular permeability to insulin could be the major underlying cause of the phenomenon and the evidence from other workers has been cited, with particular reference to insulin's delayed transit from blood to lymph, and to the impaired filtration of insulin from the forearm vascular compartment of obese or diabetic subjects. Alternative theories that have been suggested include the increased protein-binding of insulin, or the presence of biologically inactive insulin-like substances, or the circulation of specific insulin antagonists. The less convincing evidence that supports the latter hypotheses has been reviewed. Finally, the possibility that target tissue sensitivity to insulin varies sufficiently to account for the wide range of insulin levels has had little investigation. Muscle and adipose tissue could vary in their response to insulin but the liver may be a target tissue of considerable importance. Its role in glucose and insulin homeostasis has been difficult to define clearly.

An impaired secretory response of the pancreas, rather than increased insulin resistance, was evident when the "one hour" blood-sugar levels exceeded 180 mg/100 ml. The cause of defective beta cell function in diabetes has not yet been elucidated. The predictable decline of serum-insulin values above this apparent threshold invited two explanations. First, the severity of diabetes, as indicated by the height of the blood-sugar levels, may obviously have been related to the degree of insulin lack. The need to identify factors causing, or related to, the impaired secretion of insulin is thereby emphasized. The alternative explanation for the association found between insulin lack and hyperglycaemia is that high blood-sugar levels per se produce beta cell unresponsiveness. In

exploring this possibility, a significant negative association was found, in the females of the population study, between plasma osmolality and serum-insulin. It is tentatively suggested that the effect of hyperosmolality on beta cell function could contribute, at least in part, to impaired insulin secretion; for in addition to the above observation, certain hyperosmolar syndromes encountered clinically appear to be associated with temporary insulin lack. Much further investigation of factors that possibly influence insulin release is clearly necessary. The data as presented, however, bring into some clearer perspective the dual roles of insulin resistance and of impaired insulin secretion in relation to hyperglycaemia. The aetiology of diabetes will not be established until mechanisms for both phenomena have been elucidated.

In attempts to identify the pre-diabetic state, tendencies to "insulin resistance" and impaired pancreatic secretion should perhaps be separately evaluated. The only clue in this regard from the population study data was that females with a close family history of diabetes showed a consistent tendency to exhibit elevated serum-insulin responses. High insulin levels, therefore, may characterize potentially diabetic individuals, but clearly prospective studies are essential to confirm this possibility. The identification of subjects more susceptible to pancreatic beta cell failure would appear to depend on the more provocative tests of insulin secretory capacity such as massive intravenous glucose loading.

INSULIN AND ATHEROSCLEROSIS.

Elevated insulin levels have been shown to be associated with diseases of blood vessels, including essential hypertension, peripheral vascular disease, and coronary heart disease. A close relationship in the

natural population has also been demonstrated between hyperinsulinism and many concomitants of vascular disease including hyperglycaemia, hypertriglyceridaemia, obesity and ageing. Insulin thus is intimately linked with prevailing "risk factors". Mean serum-insulin levels show an early and more steep rise with age in coronary heart disease and hypertensive groups than in the general population; whereas lean, normoglycaemic subjects without vascular disease have steady mean insulin levels that do not increase until late middle age. As a characteristic of population samples therefore, low insulin levels appear to provide an index of health, whereas high levels denote vascular ageing or a measure of the prevailing metabolic disorder of urbanized communities, that may eventually become manifest as cardiovascular disease or diabetes. The findings provide epidemiological data compatible with the hypothesis that insulin is an early and direct aetiological agent in the genesis of atherosclerosis. Evidence from animal studies, in which insulin appears to contribute to, or maintain, experimental atheromata, have been reviewed; and recent "in vitro" studies of vascular tissue, which show that insulin promotes lipid accumulation in blood vessels, give collateral support to the theory. Insulin's suggested role in atherogenesis requires clear definition, both from further laboratory studies and also from prolonged prospective observation of human populations. Insulin could be one of the major links between initiating factors, such as inappropriate diet and sedentary activity and the recognized physical and biochemical manifestations of atherosclerosis. If proved, this hypothesis will provide a basis for planning more suitable preventive measures.



IMAGING SERVICES NORTH

Boston Spa, Wetherby

West Yorkshire, LS23 7BQ

www.bl.uk

Page missing in original

APPENDIX 1

MULTIPLE REGRESSION ANALYSIS USED IN THESE STUDIES*

When biological measurements show many complex inter-relationships, it is sometimes important to identify the independent associations between two variables while excluding the interfering effects of all other variables. Multiple regression analysis provides an extremely useful and elegant technique for defining the independent contribution of each of a number of factors to a particular variable in question. The technique also enables the expression of a large number of observations as a simple equation, thus summarizing or characterizing the data as a single mathematical **statement**.

PRINCIPLES.

A linear relationship between two variables is generally expressed as:

$$Y = a + bX$$

and by convention, Y is the "dependent" variable which we wish to determine in terms of X, the "independent" variable. "a" is the intercept on the Y-axis; and "b" the regression coefficient for X that determines the slope of the regression line. Given a set of values for X and Y, the line of best fit can be calculated by the "least squares" technique: this minimizes the deviations of X from the regression line, and enables the most accurate prediction of Y in terms of X.

If the dependent variable Y is related to a large number of variables $X_1, X_2, X_3, \dots, X_p$; then by the simultaneous solution of multiple equations, using the least squares method, a final linear regression equation can be derived that has

* References: Stenhouse (1965)
 Ezekiel and Fox (1959)

the following form:-

$$Y = a + b_1 X_1 + b_2 X_2 + b_3 X_3 + \dots + b_p X_p$$

where "a" is again the intercept on the Y axis, and " $b_1, b_2, b_3, \dots, b_p$ " are the regression coefficients for the independent variables $X_1, X_2, X_3, \dots, X_p$. The contribution of each variable (that is, whether its "effect" differs from zero) can be assessed by Student's "T" test, and an appropriate significance levels assigned.

In the multiple regression analysis programme used for this study (Stenhouse 1965), variables with no significant contribution (i.e. with no more effect than a random variable $p < 0.05$) are deleted one at a time, and the multiple regression equation then recalculated. This procedure is repeated until all of the independent variables remaining achieve significance (i.e. $p < 0.05$). These variables, in the final regression equation, are called "determining variables".

The coefficient of multiple correlation, R, gives a measure of the overall contribution of the determining variables to the dependent variable. For practical interpretation, the square of the coefficient of multiple correlation, R^2 , provides an estimate of the proportion of the variability of the dependent variable that is accounted for by the multiple regression equation. (Thus if R was found to be 0.70, then 0.49, or 49%, of the variability of Y is explained). However, R^2 is subject to the bias of over-estimation when many variables and complex curves are involved. R^2 can be adjusted to give an unbiased estimate; the adjusted measure is designated "A". In the text of this thesis "A" is variously referred to as

- (i) the "unbiased" index of determination; or
- (ii) the proportion of variability (of the dependent variable) ascribable to the regression.

Curvilinear functions between two variables X and Y can suitably be expressed in the following manner:-

$$Y = a + b_1 X + b_2 X^2 + b_3 X^3$$

Thus when the possibility of a non-linear relationship is anticipated in multiple regression analysis, inclusion of the square and the cube as further additional independent variables will enable a curvilinear relationship if significant to emerge in the final equation.

(Technical note: For the most accurate calculation of curvilinear functions by multiple regression analysis, the values for variables are mathematically transformed before calculation to render the mean value close to zero and the extreme values close to - 1.0 and + 1.0. Although the regression coefficients obtained can then be converted so as to be appropriate to the determining variables in their common form, it has not been possible to convert the Student "T" values. Thus, for the non-linear functions, the "T" values may not strictly apply to the corresponding regression coefficients, but are nevertheless quoted to give an indication of the overall significance of the contribution of such variables.)

APPENDIX IIRELATIONSHIP BETWEEN SERUM-INSULIN AND
BLOOD-SUGAR LEVELS IN HYPERGLYCAEMIC SUBJECTS

In lean healthy subjects with normal glucose tolerance tests, a close relationship was demonstrated between $\log_{10}$ serum-insulin values and the blood-sugar levels (Chapter 3). It was of interest to determine whether any association existed between these variables in subjects with blood-sugar levels beyond the normal range. The information was examined from 7 obese diabetic patients (Chapter 5), 9 hypertensive "diabetics" on long-term benzothiadiazime therapy (Chapter 4). In addition, blood samples were available from routine hospital glucose tolerance tests on 7 patients with coronary or peripheral vascular disease, and 9 miscellaneous (generally obese) patients: subjects in the latter two groups were "hypaglycaemic", i.e. the one hour blood-sugar level exceeded 180 mg/100 ml. or the two hour level exceeded 115 mg/100 ml.

The accompanying figure shows the relationship found between serum-insulin (on a log-scale) and blood-sugar levels during standard 50 g. oral glucose tolerance tests. The sloping parallel lines indicate the mean and 95% confidence limits for the regression of blood-sugar on $\log_{10}$ serum-insulin from the 45 normal subjects (Chapter 3).

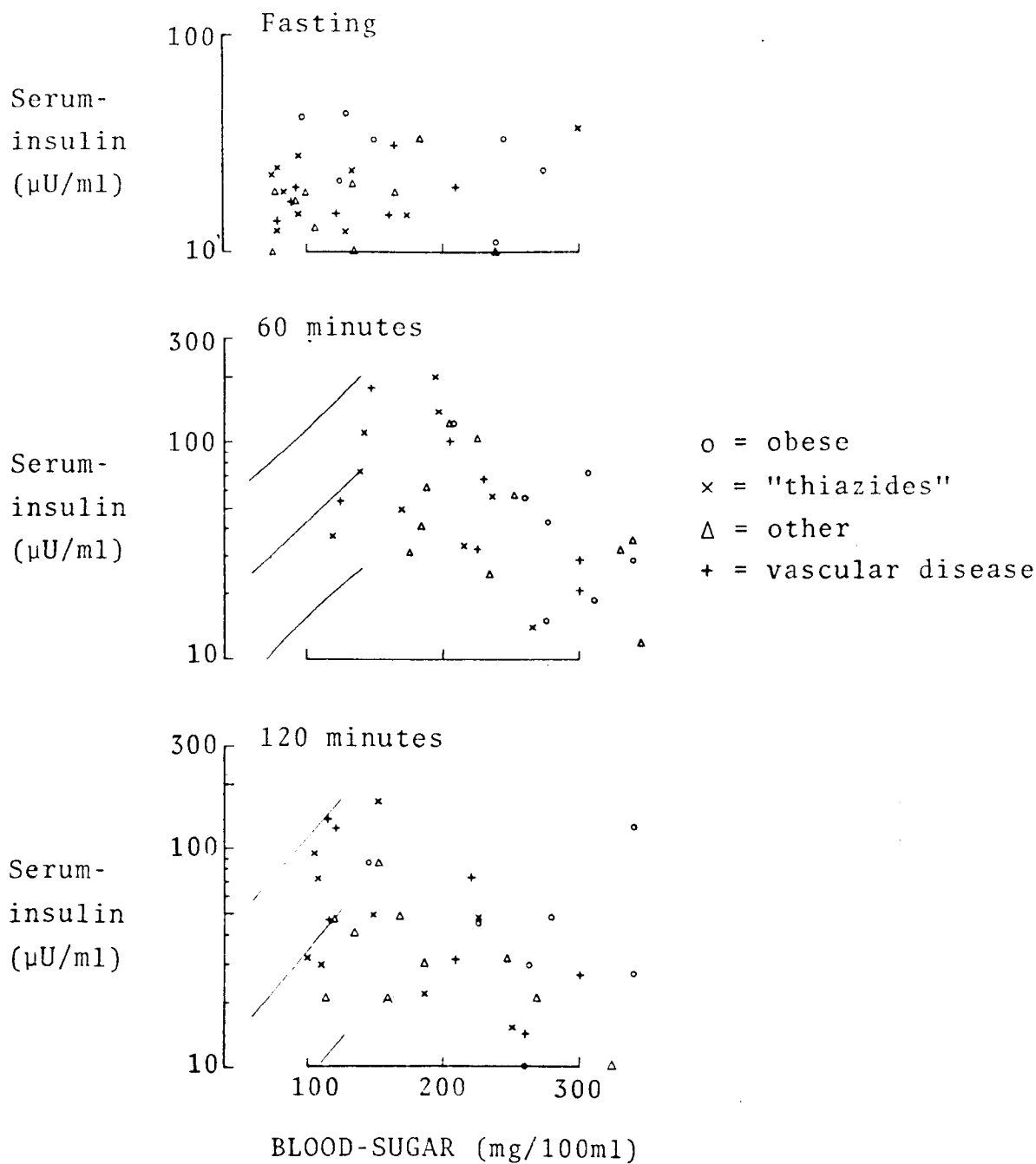
The fasting serum-insulin levels of the hyperglycaemic subjects were generally elevated, but showed no correlation with blood-sugar. At one hour after glucose, the range of insulin values obtained in hyperglycaemic subjects corresponded to the range found

in the normal subjects. When related to the blood-sugar levels, however, a distinct negative association between serum-insulin and increasing hyperglycaemia became apparent, and the abnormal insulin responses of diabetics was more clearly demonstrated. The scatter of insulin values, for a given blood-sugar level, in the hyperglycaemic subjects was no greater than in the normal subjects. The decline in insulin levels was evident when blood sugar levels exceeded 180-200 mg/100 ml. At two hours, a similar trend occurred but was less well seen.

The one hour test, therefore, appeared to provide the best discrimination between the high insulin levels of mild hyperglycaemia and the impaired insulin responses of the more severe diabetics.

SERUM-INSULIN VALUES

FOR MISCELLANEOUS DIABETICS



REFERENCES

- Abel, J.J. (1926) Crystalline insulin. Proc. Acad. Med. Sci., 12, 132.
- Abell, L.L., Levy, B.B., Brodie, B.B. et al. (1952) Simplified method for estimation of total cholesterol in serum. J. biol. Chem., 195, 357.
- Abrams, M.E., Jarrett, R.J., Keen, H., et al. (1969) Oral glucose tolerance and related factors in a normal population sample. II. Brit. med. J., 1, 599.
- Albrink, M.J. (1965) Carbohydrate metabolism in cardiovascular disease. Ann. int. Med., 62, 1330.
- Albrink, M.J., Meigs, J.W. and Man, E.B. (1961) Serum lipids, hypertension, and coronary artery disease. Amer. J. Med., 31, 4.
- Alp, H. and Recant, L. (1963) Studies of the insulin inhibitory effect of human albumin fractions. J. clin. Invest., 44, 870
- Anderson, E. and Long, J.A. (1947) The effect of hyperglycaemia on insulin secretion with the isolated rat pancreas. Endocrinology, 40, 92.
- Anderson, E., Lindner, E. and Sutton, V. (1947) A sensitive method for the assay of insulin in blood. Amer. J. Physiol., 149, 350.
- Antoniades, H.N. (1961) Studies on state of insulin in blood. Endocrinology, 68, 7.
- Antoniades, H.N., Bougas, J. and Pyle, H.M. (1962) Studies on the state of insulin in blood. New Eng. J. Med., 267, 218.
- Antoniades, H.N., Gundersen, K. and Pyle, H.M. (1961) Studies on the state of insulin in blood. Endocrinology, 69, 163.
- Arendt, E.C. and Patee, C.J. (1956) Studies on obesity. The insulin-glucose tolerance curve. J. clin. Endocr., 16, 367.
- Arky, R.A. and Freinkel, N. (1966) Alcohol hypoglycaemia (to test gluconeogenesis in starvation). New Eng. J. Med., 274, 426.
- Armstrong, J., Bornstein, J., Ng, F. et al. (1969) Pituitary polypeptide with hypoglycaemic action in diabetes mellitus. Brit. med. J., 2, 157.

- Arquilla, E.R. and Stavitsky, A.B. (1956) The production and identification of antibodies to insulin and their use in assaying insulin. *J. clin. Invest.*, 35, 458.
- Arquilla, E.R., Ooms, H. and Mercola, K. (1968) Immunological and biological properties of iodo-insulin labelled with one or less atoms of iodine per molecule. *J. clin. Invest.*, 47, 474.
- Bagdade, J.D., Bierman, E.L. and Porte, D. (1967) The significance of basal insulin levels. *J. clin. Invest.*, 46, 1549.
- Baird, C.W. and Bornstein, J. (1957) Plasma-insulin and insulin resistance. *Lancet*, 1, 1111.
- Baird, C.W. and Bornstein, J. (1959) Assay of Insulin-like activity in the plasma of normal and diabetic human subjects. *J. Endocr.*, 19, 74.
- Ball, E.G. and Jungas, R.L. (1964) Some effects of hormones on the metabolism of adipose tissue. *Rec. Progr. Horm. Res.*, 20, 183.
- Balsano, F., Pitucco, G., Musca, A. et al. (1964) New interpretation of oral glucose tolerance. *Lancet*, 2, 865.
- Banal, P. and Roux, J. (1931) Anaphylactic responses to insulin. *Compt. Rend. Soc. Biol.*, 106, 292.
- Banerjee, R.N. (1962) A methodological study of the immunoassay of human insulin by an isotopic tracer technique. Ph.D. Thesis, London University.
- Banting, F.G. and Best, C.H. (1922) The internal secretion of the pancreas. *J. Lab. clin. Med.*, 1, 251.
- Banting, F.G., Best, C.H., Colip, J.B. et al. (1922) The effect of pancreatic extracts on normal rabbits. *Amer. J. Physiol.*, 62, 162.
- Banting, F.G., Frank, W.R. and Gairns, S. (1938) Anti-insulin activity of serum of insulin treated patients. *Amer. J. Psychiat.*, 93, 562.
- Beck, L.V., Roberts, N., Blankenbahn, R. et al. (1966) On insulin immunologic activity in livers and kidneys of mice infected with beef insulin. *Proc. Soc. exp. Biol. Med.*, 122, 768.
- Beck, L.V., Zaharko, D.S., Roberts, N. et al. (1966) On insulin- I^{131} metabolism in mice: modifying effects of anti-insulin serum and of total insulin dosage. *Diabetes*, 15, 336.

- Beck, P., Koumans, J., Winterling, C. et al. (1964) Studies of insulin and growth hormone secretion in human obesity. *J. Lab. clin. Med.*, 64, 654.
- Beigelman, P.M. (1958) Insulin-like activity of serum protein fractions. *Diabetes*, 7, 365.
- Berkowitz, D. (1964) Blood lipid and uric acid interrelationships. *J.A.M.A.*, 190, 856.
- Bernard, C., (1859) *Lecons sur le diabete et la glycogenese animale*. Paris.
- Berson, S.A. and Yalow, R.S. (1957) Radiochemical and radio biological alterations of I^{131} -labelled proteins in solution. *Ann. N.Y. Acad. Sc.*, 70, 56.
- Berson, S.A. and Yalow, R.S. (1958) Isotopic tracers in the study of diabetes. *Adv. Biol. Med. Phys.*, 6, 349.
- Berson, S.A. and Yalow, R.S. (1959) Quantitative aspects of reactions between insulin and insulin binding antibody. *J. clin. Invest.*, 38, 1996.
- Berson, S.A. and Yalow, R.S. (1959) Species-specificity of human anti-beef port insulin serum. *J. clin. Invest.*, 38, 2017.
- Berson, S.A. and Yalow, R.S. (1959) Recent studies on insulin-binding antibodies. *Ann. N.Y. Acad. Sci.*, 8, 338.
- Berson, S.A. and Yalow, R.S. (1961) Preparation and purification of human insulin-I : binding to insulin-binding antibodies. *J. clin. Invest.*, 40, 1803.
- Berson, S.A. and Yalow, R.S. (1962) Immunoassay of plasma insulin. *Ciba Found. Colloq. Endocr.*, 15, 182.
- Berson, S.A. and Yalow, R.S. (1964) The present status of insulin antagonists in plasma. *Diabetes*, 13, 247.
- Berson, S.A. and Yalow, R.S. (1965) Some current controversies in diabetes research. *Diabetes*, 14, 549.
- Berson, S.A., Yalow, R.S. Bauman, A. et al. (1956) Insulin-I metabolism in human subjects; demonstration of insulin-binding globulin in the circulation of insulin-treated subjects. *J. clin. Invest.*, 35, 170.
- Berson, S.A., Yalow, R.S., Glick, S.M. et al. (1964) Immunoassay of protein and peptide hormones. *Metabolism*, 13, 1135.

- Best, C.H., Haist, R.E. and Ridout, J.H. (1939) Diet and insulin content of pancreas. *J. Physiol.*, 97, 107.
- Blackburn, H., Keys, A., Simonsen, E. et al. (1960) The ECG in population studies. A classification system. *Circulation*, 21, 1160.
- Boden, G. and Soeldner, J.S. (1967) A sensitive double antibody radioimmunoassay for HGH. *Diabetologia*, 3, 413.
- Bolinger, R.E., Brown, R.W. and Lukert, B.P. (1965) Insulin-like activity in urine of normal and obese subjects. *Metabolism*, 14, 568.
- Bolinger, R.E., van der Geld, H. and Willebrands, A.F. (1959) Electrophoretic separation of insulin activity of normal plasma and plasma with added insulin-I¹³¹. *Metabolism*, 8, 39.
- Bornstein, J. (1950) A technique for the assay of small quantities of insulin using ADHA rats. *Aust. J. exp. Biol. Med. Sci.*, 28, 87.
- Bornstein, J. (1953) Insulin content of blood plasma. *Diabetes*, 2, 23.
- Bornstein, J., Armstrong, J., Ng, F. et al. (1969) Potentiation of insulin action in normal subjects by a pituitary polypeptide. *Brit. med. J.*, 2, 451..
- Bornstein, J. and Lawrence, R.D. (1951) Two types of diabetes mellitus with and without available plasma insulin. *Brit. med. J.*, 1, 732.
- Boyns, D.R., Jarrett, R.S. and Keen, H. (1967) Intestinal hormones and plasma insulin: an insulintropic action of secretin. *Brit. med. J.*, 2, 676.
- Boyns, D.R., Crossley, J.N., Abrams, M.E. et al. (1969) Oral glucose tolerance and related factors in a normal population survey. I. *Brit. med. J.*, 1, 595.
- Breckenridge, A. (1966) Hypertension and hyperuricaemia. *Lancet*, 1, 15.
- Brunfeld, K. and Deckert (1968) Aminoacid content of human crystalline insulin from non-diabetic and diabetic patients. *Acta endocrin.*, 60, 543.

Brunfeldt, K. and Jorgensen, K. (1967) Some factors of significance in the double antibody immunoassay of insulin. *Acta endocr.*, 54, 347.

Buchanan, K.D. and McKiddie, M.T. (1967) Experience with the immunoprecipitation technique of insulin assay. *Clin. chim. Acta*, 15, 315.

Burman, D. and Glaspole, D. (1964) Alimentary hyperglycaemia simulating diabetic ketosis. *Lancet*, 1, 208.

Butterfield, W.J.H. (1965) In *On the Nature and Treatment of Diabetes*. Eds. Liebel and Wrenshall. p.768. Excerpta Medica Foundation, Amsterdam, The Netherlands.

Butterfield, W.J.H. (1967) In *Third Symposium on Advanced Medicine*, p. 280. Ed. A.M. Dawson. Pitman Medical.

Butterfield, W.J., Abrams, M.E., St. John, D.J. et al. (1967) The intravenous glucose tolerance test: peripheral disposal of the glucose load in controls and diabetics. *Metabolism*, 16, 19.

Butterfield, W.J.H., Garrett, C.J. and Whichelow, M.J. (1963) Peripheral hormone action: studies on the clearance and effect of (¹³¹-I) iodoinsulin in the peripheral tissues of normal acromegatic and diabetic subjects. *Clin. Sci.*, 24, 331.

Cahill, G.F., Ashmore, J., Earle, A.S. et al. (1958) Glucose penetration into liver. *Amer. J. Physiol.*, 192, 491.

Cahill, G.F., Herrera, M.G., Morgan, A.P. et al. (1966) Hormone-fuel interrelationships during fasting. *J. clin. Invest.*, 45, 1751.

**

Canellos, G.P. and Hatch, F.T. (1962) Metabolic studies in young males with coronary heart disease. *Circulation*, 26, 648.

Caraway, W.T. (1955) Determination of uric acid in serum by a carbonate method. *Amer. J. clin. Path.*, 25, 840.

Carlson, L.A. (1960) Serum lipid in man with myocardial infarction. *Acta med. scand.*, 167, 399.

Catt, K.J., Cain, M.D., Zimmet, P.Z. et al. (1969) Blood angiotensin II levels of normal and hypertensive subjects. *Brit. med. J.*, 1, 819.

**

Camerini-Davalos, R., Canefield, J., Rees, S. et al. (1963) Preliminary observations on subjects with prediabetes. *Diabetes*, 12, 508.

- Catt, K., Niall, H.D. and Taguear, G.W. (1966) Solid-phase radioimmunoassay of human growth hormone. *Biochem. J.*, 100, 31-33c.
- Cerasi, E. and Luft, R. (1963) Plasma insulin response to sustained hyperglycaemia induced by glucose infusion in human subjects. *Lancet*, 2, 1359.
- Cerasi, E. and Luft, R. (1967) The plasma insulin response to glucose infusion in healthy subjects and in diabetes (and in prediabetes). *Acta Endocr.*, 55, 278, 305, 330.
- Chamberlain, M.J. and Stimmler, L. (1967) The renal handling of insulin. *J. clin. Invest.*, 46, 911.
- Chao, P.Y., Karam, J.H. and Grodsky, C.M. (1965) Insulin- I^{131} binding in serum from normal and diabetic subjects by ultracentrifugation and gel filtration. *Diabetes*, 14, 27.
- Chlouverakis, C., Jarrett, R.J. and Keen, H. (1967) Glucose tolerance, age, and circulating insulin. *Lancet*, 1, 806.
- Christiansen, K., Deckert, T., Kjerulf, K. et al. (1968) Glucose tolerance, plasma lipids and serum insulin in patients with ischaemic heart diseases. *Acta med. scand.*, 184, 283.
- Christensen, N.J. and Orksov, H. (1968) The relationship between endogenous serum insulin concentration and glucose uptake in the forearm muscles of non-diabetics. *J. clin. Invest.*, 47, 1262.
- Clausen, J., Gjedde, F. and Gorgensen, K. (1963) Insulin binding proteins in human serum. *Proc. Soc. exp. Biol. Med.*, 112, 778.
- Cohen, H. (1958) 17-ketogenic steroid excretion in obese children before and after weight reduction. *Brit. med. J.*, 1, 686.
- Cohen, A.M. and Shafrir, E. (1965) Carbohydrate metabolism in myocardial infarction. *Diabetes*, 14, 84.
- College of General Practitioners' Working Party (1962) "A diabetes survey". *Brit. med. J.*, 1, 1497.
- Conn, J.W. (1965) Hypertension, the potassium ion, and impaired carbohydrate tolerance. *New Eng. J. Med.*, 273, 1135.

- Conn, J.W., Louis, L.H. and Johnston, M.M. (1949) Metabolism of uric acid - during induction of diabetes in man with ACTH. *J. Lab. clin. Med.*, 34, 255.
- Crockford, P.M., Harbeck, R.J. and Williams, R.H. (1966) Influence of age on intravenous glucose tolerance and serum immunoreactive insulin. *Lancet*, 1, 465.
- Crockford, P.M., Porte, D., Wood, F.C. et al. (1966) Effect of glucagon on serum insulin, plasma glucose, and free fatty acids in man. *Metabolism*, 15, 114.
- Danowski, T.S. (1969) Evolution of childhood and adult types of diabetes from pre-diabetes. Banting Memorial Lecture. Presented at British Diabetic Association Meeting, London, October 1969.
- Danowski, T.S. and Nabarro, J.D.N. (1965) Hyperosmolar and other types of non-ketoacidotic coma in diabetes. *Diabetes*, 14, 162.
- Dash, J.A. and Lindsay, D.B. (1967) Comparison of an immunoassay and bioassay of insulin in sheep plasma. *J. Endocr.*, 37, 119.
- Davidson, P.C. and Albrink, M.J. (1965) Insulin resistance in hyperglyceridemia. *Metabolism*, 14, 1059.
- Davidson, P.C. and Albring, M.J. (1966) Insulin resistance in hyperglyceridaemia. *J. clin. Invest.*, 45, 1000.
- Dawber, T., Moore, F. and Mann, G. (1957\$) Coronary heart disease in the Framingham study. *Amer. J. Pub. Health.*, 47, (suppl. 4), 4.
- de Bodo, R.C., Steele, R., Altszuler, N. et al. (1959) Further studies on the mechanism of action of insulin. *Metabolism*, 8, 520.
- de Meyer, J. (1909) Action de la secretion interne du pancreas. *Arch. di Fisiol.*, 7, 96.
- Diem, K. (1962) *Documenta Geigy Scientific Tables*. 6th Ed. p. 624. Geigy, Basle, Switzerland.
- Ditzel, J. and St. Clair, R.W. (1954) Clinical photography ... of bulbar conjunctiva. *Circulation*, 10, 277.

- Dollery, C.T., Breckenridge, A., Welborn, T. et al (1965) Diabetogenic effect of benzothiadiazine diuretics: assessment of risk.
- Dollery, C.T., Pentecost, B.L. and Samaan, N.A. (1962) Drug-induced diabetes. *Lancet*, 2, 735.
- Duff, G.L. and McMillan, G.C. (1949) Effect of alloxan diabetes on experimental cholesterol atherosclerosis in rabbit. *J. exp. Med.*, 89, 611.
- Duncombe, W.G. (1964) The colorimetric micro-determination of NEFA in plasma. *Clin. chim. Acta*, 9, 122.
- Dupre, J., Curtis, J.D., Unger, R.M. et al. (1969) Effects of secretin, pancreozymin, or gastrin on the response of the endocrine pancreas. *J. clin. Invest.*, 48, 745.
- Egdahl, R.H. and Goldberg, H. (1962) Pancreatic and hepatic influences on serum insulin-like activity in the dog. *Surg. Gynec. Obst.*, 114, 202.
- Elgee, N.J. and Williams, R.H. (1954) Degradation of insulin- I^{131} by liver and kidney in vivo. *Proc. Soc. exp. Biol. Med.*, 87, 352.
- Elgee, N.J., Williams, R.H. and Lee, N.D. (1954) Distribution and degradation studies with insulin- I^{131} . *J. clin. Invest.*, 33, 1252.
- Ellenberg, M. (1963) Diabetic complications without manifest diabetes. *J.A.M.A.*, 183, 926.
- Elliott, R.B., O'Brien, D. and Roy, C.C. (1965) An abnormal insulin in diabetes mellitus. *Diabetes*, 14, 780.
- Elrick, H.L., Stimmler, L., Hlad, C.J. et al. (1964) Plasma insulin response to oral and intravenous glucose. *J. clin. Endocr.*, 14, 1076.
- Ensinck, J., Mahler, R. and Vallance-Owen, J. (1965) Antagonism of insulin action on muscle by the albumin-bound B chain of insulin. *Biochem. J.*, 94, 150.
- Epstein, F.V.H., Ostrander, L.D., Johnson, B.C. et al. (1965) Epidemiological studies of cardiovascular diseases in a total community - Tecumseh, Michigan. *Ann. int. Med.*, 62, 1170.

- Ezekiel, M. and Fox, K.A. (1959) Methods of correlation and regression analysis. John Wiley and Sons Inc.
- Fajans, S.S., Floyd, J.C., Knopf, R.F. et al. (1966) Benzo-thiadiazine suppression of insulin release from normal and abnormal islet tissue in man. J. clin. Invest., 45, 481.
- Farquhar, J.W., Frank, A., Gross, R.C. et al. (1966) Glucose, insulin and triglyceride response to high and low carbohydrate diets in man. J. clin. Invest., 45, 1648.
- Fenton, E.L. (1959) A method for the assay of insulin by paper chromatography. Biochem. J., 71, 507.
- Fenebee, J.W. (1951) Insulin and ACTH labelled with radio-iodine. Endocrinology, 48, 277.
- Floyd, J.C., Fajans, S., Knopf, R.F. et al. (1964) Evidence that insulin release is the mechanism for leucine hypoglycaemia. J. clin. Invest., 42, 1714.
- Franckson, J.R.M., Malaisse, W., Arnould, Y. et al. (1966) Glucose kinetics in human obesity. Diabetologia, 2, 96.
- Fraser, R. and Hartog, M. (1962) Immunological studies with human growth hormone. Ciba Found. Colloq. Endocr., 14, 105.
- Fraser, R., Joplin, G.F., Opie, L.H. et al. (1962) The augmented insulin tolerance test for detecting insulin resistance. J. Endocr., 25, 299.
- Fraser, R. and Wright, A.D. (1968) Standard procedures for assessing human growth hormone, using the radio-immunoassay. Postgrad. Med. J., 44, 53.
- Froesch, E.R., Burgi, H., Ramserer, E.B. et al. (1963) Antibody-suppressible and non-suppressible insulin-like activity in human serum and their physiologic significance. J. clin. Invest., 42, 1816.
- Froesch, E.R., Burki, H., Muller, W.A. et al. (1967) Non-suppressible insulin-like activity of human serum. Rec. Progr. Horm. Res., 23, 565.
- Garcia, J., Linfoot, J.A., Marrougian, E. (1967) Plasma growth hormone studies in normal individuals and acromegalics. J. clin. Endocr., 27, 1395.
- Gellhorn, J., Feldman, J. and Allen, A. (1941) Assay of insulin on hypophysectomized rats. Endocrinology, 29, 137.

- Gener-Segui, J. and Wolfe, A.J. (1966) Studies of "bound" insulin by epididymal fat pad assay. *Diabetes*, 15, 894.
- Genuth, S.M. (1966) Effects of prolonged fasting on insulin secretion. *Diabetes*, 15, 798.
- Genuth, S.M., Frohman, L. and Lebovitz, H.E. (1965) A radio-immunological assay method for insulin using gel filtration. *J. clin. Endocr.*, 25, 1043.
- Goetz, F.C., Marrey, J.W. and Greenberg, B.Z. (1967) Regulation of insulin secretion: effects of infusion of ... sugars into portal veins of dogs. *J. Lab. clin. Med.*, 69, 537.
- Goldberg, G. and Egdahl, H.L. (1961) Serum insulin-like activity in pancreatectomized dogs. *Surg. Forum*, 12, 360.
- Gorden, P. and Roth, J. (1969) Circulating insulins. *Arch. int. Med.*, 123, 237.
- Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) The preparation of I^{131} -labelled HGH of high specific radioactivity. *Biochem. J.*, 89, 114.
- Grodsky, G.M. and Forsham, P.H. (1960) An immunochemical assay of total extractible insulin in serum. *J. clin. Invest.*, 39, 1070.
- Grodsky, G.M., Batts, A.A., Bennett, L.L. et al. (1963) Effects of carbohydrates on secretion of insulin from isolated rat pancreas. *Amer. J. Physiol.*, 205, 638.
- Grodsky, G.M., Karam, J.H., Pavlatos, F. et al. (1965) Serum-insulin response to glucose in prediabetic subjects. *Lancet*, 1, 290.
- Groen, J., Kamminger, C.E., Willebrands, H. et al. (1952) Evidence for the presence of insulin in blood serum. A method for the approximate determination of the insulin content of blood. *J. clin. Invest.*, 31, 97.
- Hales, C.N. (1964) Plasma insulin in diabetes. *Ciba Found. Colloq. Endocr.*, 15, 140.
- Hales, C.N. and Randle, P.J. (1963) Immunoassay of insulin with insulin-antibody precipitate. *Biochem. J.*, 88, 137.
- Hales, C.N. and Randle, P.J. (1963) Effect of low carbohydrate diets on plasma concentrations of glucose, NEFA, and insulin during oral GTT. *Lancet*, 1, 790.

- Hales, C.N., Greenwood, F.C., Mitchell, F.L. et al. (1968) Blood-glucose, plasma insulin and growth hormone concentration of individuals with minor abnormalities of glucose tolerance. *Diabetologia*, 4, 73.
- Hales, C.N., Walker, J.B., Garland, P.B. et al. (1965) Fasting plasma concentrations of insulin- non-esterified fatty acids, glycerol, and glucose in the early detection of diabetes mellitus. *Lancet*, 1, 65.
- Hall, A.P. (1964) Serum uric acid and coronary heart disease. *Arthr. Rheum.*, 8, 846.
- Hartog, M., Gaafar, M., Meisser, B. et al. (1964) Immunoassay of serum growth hormone in acromegalic patients. *Brit. med J.*, 2, 1229.
- Havel, R.J. (1961) Conversion of plasma free fatty acids into tryglycerides of plasma lipoprotein fractions in man. *Metabolism*, 10, 1031.
- Hayes, T.M. and Woods, C.J. (1968) Hyperosmolar non-ketotic coma. *Lancet*, 1, 209.
- Hayner, N.S., Kjelsberg, M.O., Epstein, F.H. et al. (1965) Carbohydrate tolerance and diabetes in a total community - Tecumseh, Michigan. *Diabetes*, 14, 413.
- Hayner, N.S., Waterhouse, A.M. and Gordon, T. (1963) Vital and Health Statistics Series 2, No. 3. "The one-hour glucose tolerance test". U.S. Government Printing Office, Washington.
- Herbert, V., Lau, K.S., Gottlieb, C.W. et al. (1965) Coated charcoal immunoassay of insulin. *J. clin. Endocr.*, 25, 1375.
- Himsworth, H.P. (1935) Dietetic factor determining glucose tolerance and sensitivity to insulin of healthy men. *Clin. Sci.*, 2, 67.
- Hoffman, W.S. (1937) Rapid photoelectric method for the determination of glucose in blood and urine. *J. biol. Chem.*, 120, 51.
- Humbel, R.E. (1965) Biosynthesis of the two chains of insulin. *Proc. nat. Acad. Sci.*, 53, 853.
- Hunter, W.M. and Greenwood, F.C. (1962) Preparation of iodine-I¹³¹ labelled human growth hormone of high specific activity. *Nature*, 194, 495.

- Izzo, J.L., Bale, W.F., Izzo, M.J. et al. (1964) High specific activity labeling of insulin with ^{131}I . J. biol. Chem., 239, 3743.
- Izzo, J.L., Roncoue, A., Izzo, M.J. et al. (1964) Relationship between degree of iodination of insulin and its biological electrophoretic, and immunochemical properties. J. biol. Chem., 239, 3749.
- Jackson, W.P.U. and Forman, R. (1966) Hyperosmolar nonketotic diabetic come. Diabetes, 15, 714.
- Jackson, I.M., McKiddie, M.T. and Buchanan, K.D. (1969) Effect of fasting on glucose and insulin metabolism of obese patients. Lancet, 1, 285.
- Jarrett, R.J. and Keen, H. (1969) Diurnal variation of oral glucose tolerance: a possible pointer to the evolution of diabetes mellitus. Brit. med. J., 2, 341.
- Jenkins, D.J.A. (1967) Ketone bodies and the inhibition of free fatty acid release. Lancet, 2, 338.
- Johansen, K. and Lunbaek, K. (1967) Plasma insulin in mild juvenile diabetes. Lancet, 1, 1257.
- Johansen, K. and Orksov, K. (1969) Plasma insulin during remission in juvenile diabetes mellitus. Brit. med. J., 1, 676.
- Jorgensen, K.R. (1969) Evaluation of the double antibody radioimmunoassay of insulin ... in plasma of normal subjects. Aeta Endocrin., 60, 327.
- Joslin, E.P., Dublin, L.I. and Marks, H.H. (1936) Studies in diabetes mellitus. Amer. J. Med. Sci., 192, 9.
- Joslin, E.P., Root, H.F., White, P. et al. (1959) In The Treatment of Diabetes Mellitus. Lee & Febiger, Philadelphia.
- Karam, J.H., Grodsky, G.M. and Forsham, P.H. (1963) Excessive insulin response to glucose in obese subjects as measured by immunochemical assay. Diabetes, 12, 197.
- Karam, J.H., Grodsky, G.M. and Forsham, P.H. (1965) The relationship of obesity and growth hormone to serum insulin levels. Ann. N.Y. Acad. Sci., 131, 374.

- Karam, J.H., Grodsky, G.M., Pavlatos, F.C. et al. (1965) Critical factors in excessive serum insulin response to glucose: obesity in maturity onset diabetics and growth hormone in acromegaly. *Lancet*, 1, 286.
- Katzen, H.M., Tietze, F. and Stetten, D. (1963) Further studies on the properties of hepatic glutathion-insulin trans-hydrogenase. *J. biol. Chem.*, 238, 1006.
- Keen, H., Rose, G., Pyke, D.A. et al. (1965) Blood sugar and arterial disease. *Lancet*, 2, 505.
- Kemsley, W. (1951) Height and weight tables. *Ann. Eugen.* Lond., 16, 316.
- Kessler, J.I. (1962) Effect of insulin on release of plasma lipolytic activity. *J. Lab. clin. Med.*, 60, 747.
- Kreizberg, R.A., Boshell, B.R., DiPlacido, J. et al. (1967) Insulin secretion in obesity, *New Eng. J. Med.*, 276, 314.
- Kumar, R.S. (1968) Hyperosmolar non-ketotic coma. *Lancet*, 1, 48.
- Kuzuya, T. and Samols, E. (1964) The plasma insulin "inhibitor" and immunoprecipitation assay. *Metabolism*, 13, 493.
- Kuzuya, T., Samols, E. and Williams, R.H. (1965) Stimulation by hyperosmolarity of glucose metabolism in rat adipose tissue and diaphragm in vitro. *J. biol. Chem.*, 240, 2277.
- Lacey, A.H. (1967) The unit of insulin. *Diabetes*, 16, 198.
- Langdon, R.G. and Wolff, F.W. (1964) Conjoint clinic on drugs and diabetes. *J. chron. Dis.*, 17, 585.
- Lawrence, A.M. (1966) Radioimmunoassayable glucagon levels in man. *Proc. nat. Acad. Sci.*, 55, 316.
- League of Nations Health Organization Report (1926) The Biological Standardization of Insulin, Geneva.
- Lee, N.D. (1957) Studies on insulin labelled with I^{131} . *Ann. N.Y. Acad. Sci.*, 70, 94.
- Leonards, J.R. (1959) Assay of insulin-like activity. *Fed. Proc.*, 18, 272.
- Levin, S. and Geller-Bernstein, C. (1964) Alimentary hyperglycaemia simulating diabetic keto-acidosis. *Lancet*, 2, 595.

- Levine, R.A., Streeten, D.H.P. and Doisy, R.J. (1968) Effects of oral chromium supplementation on the abnormal glucose tolerance of elderly human subjects. *Metabolism*, 17, 114.
- Lofland, H.B. (1964) A semi-automated procedure for the determination of triglyceride in serum. *Anal. Biochem.*, 9, 393.
- Lovell, R.R. (1966) The relationship between blood-pressure and age in various races of mankind. *Gerontologist*, 6, 111.
- Lowy, C., Blanshard, G. and Phear, D. (1961) Antagonism of insulin by albumin. *Lancet*, 1, 802.
- Lyngsoe, J. (1962) The insulin-like activity in serum determined by the rat epididymal fat method. I. *Acta med. scand.*, 171, 365.
- Lyngsoe, J. (1962) Insulin-like activity in serum determined by the rat epididymal fat method. *Acta med. scand.*, 172, 41.
- Lyngsoe, J. (1962) The insulin-like activity in serum determined by the rat epididymal fat method. III. *Acta med. scand.*, 172, 601.
- Lyngsoe, J. (1964) The insulin-like activity in serum determined by the rat epididymal fat method. V. *Acta med. scand.*, 175, 401.
- McCall, M.G. (1967) Normality. *J. chron. Dis.*, 19, 1127.
- McGill, H.C. and Holman, R.L. (1949) Influence of alloxan diabetes on cholesterol atheromatosis in rabbit. *Proc. Soc. exp. Biol. Med.*, 72, 72.
- McIntyre, N., Holdsworth, C.D. and Turner, D.S. (1964) New interpretation of oral glucose tolerance. *Lancet*, 2, 20.
- MacKay, E.M. and Bergman, H.C. (1933) Rate of absorption of glucose from intestinal tract. *J. biol. Chem.*, 101, 453.
- Madison, L.L. and Unger, R.H. (1959) The physiologic significance of the secretion of endogenous insulin into the portal circulation. I. *J. clin. Invest.*, 37, 631.
- Madison, L.L., Mebane, D. and Unger, R.H. (1964) Hypoglycaemic action of ketones. II. *J. clin. Invest.*, 43, 408.
- Madison, L.L., Combes, B., Adams, R. et al. (1960) The physiological significance of the secretion of endogenous insulin into the portal circulation. *J. clin. Invest.*, 39, 507.

- Mahler, R. (1965) Diabetes and arterial lipids. Quart. J. Med., 34, 484.
- Mahler, R.F. (1966) In Diabetes Mellitus. Ed. L.P.J. Duncan Edinburgh.
- Malins, J. (1968) In Clinical Diabetes Mellitus. Eyre & Spottiswoode, London.
- Marsh, J.B. and Hangaard, N. (1952) Effect of serum from insulin-resistant cases on combination of insulin with rat diaphragm. J. clin. Invest., 31, 107.
- Martin, D.B., Renold, A.E. and Dagenais, Y. (1958) An assay for insulin-like activity using rat adipose tissue. Lancet, 2, 76.
- Martin, F.I.R. and Stocks, A.E. (1968) Insulin sensitivity and vascular disease in insulin-dependent diabetics. Brit. med. J., 2, 81.
- Martin, F.I.R., Pearson, M.J. and Stocks, A.E. (1968) Glucose tolerance and insulin insensitivity. Lancet, 1, 1285.
- Meade, R.C. and Klitgaard, H.M. (1962) A simplified method for immunoassay of human serum insulin. J. Nucl. Med., 3, 407.
- Meek, J.C., Doffing, K.M. and Bolinger, R.E. (1968) Radioimmunoassay of insulin A and B chains in normal and diabetic human plasma. Diabetes, 17, 16.
- Milner, R.D.G. and Hales, C.N. (1968) Cations and the secretion of insulin. Biochim. Biophys. Acta, 150, 165.
- Mirsky, I.A., Podore, C.J., Wachman, J. et al. (1948) Urinary excretion of insulin by normal and diabetic subjects. J. clin. Invest., 27, 515.
- Mitchell, F.L. and Strauss, W.T. (1964) Relation of post-prandial blood-glucose level to the oral glucose tolerance curve. Lancet, 1, 1185.
- Mitchell, J.R.A. and Schwartz, C.J. (1965) Arterial disease. Blackwell, Oxford.
- Mitchell, M.L. (1963) Interaction of radio-insulin with serum proteins in vitro and with liver in vivo. J. clin. Endocr., 23, 1001.

- Mitchell, M.L., Collins, S. and Byron, J. (1969) Radioimmunoassay of growth hormone by enzyme partition. *J. clin. Endocr.*, 29, 257.
- Moloney, P.J. and Coval, M. (1955) Antigenicity of insulin: diabetes induced by specific antibodies. *Biochem. J.*, 59, 179.
- Morgan, C.R. and Lazarow, A. (1963) Immunoassay of insulin: two antibody system. *Diabetes*, 12, 115.
- Morgan, C.R., Sorenson, R.L. and Lazarow, A. (1964) Studies of an inhibitor of the two antibody immunoassay system. *Diabetes*, 13, 1.
- Morgan, C.R., Sorenson, R.L. and Lazarow, A. (1964) Further studies of an inhibitor of the two antibody assay system. *Diabetes*, 13, 579.
- Mortimore, G.E., Tietze, F. and Stetten, D. (1959) Metabolism of insulin I^{131} : studies with isolated perfused rat liver. *Diabetes*, 8, 307.
- Neel, J.V. (1962) Diabetes mellitus: a "thrifty" genotype rendered detrimental by progress. *Amer. J. Hum. Genet.*, 14, 353.
- Nestel, P.J. (1967) Relationship between FFA flux and TGFA flux in plasma before and during the infusion of insulin. *Metabolism*, 16, 1123.
- Newell (quoted by McDonough et al.) (1965) *J. Chron. Dis.*, 18, 443.
- Nikkila, E.A., Miettinen, T.A. and Vesonne, M.R. (1965) Plasma-insulin in coronary heart disease: response to oral and intravenous glucose and to tolbutamide. *Lancet*, 2, 508.
- Nikkila, E., Taskinen, M., Miettinen, T. et al. (1968) Effect of muscular exercise on insulin secretion. *Diabetes*, 17, 209.
- Nitzan, M. and Zelmanovsky, H. (1968) Glucose intolerance in hypernatremic rats. *Diabetes*, 17, 579.
- O'Brien, J.P. and Sharpe, A.R. (1967) The influence of renal disease on the insulin- I^{131} disappearance curve in man. *Metabolism*, 16, 76.
- Ogilvie, R.F. (1933) Islands of Langerhans in 19 cases of obesity. *J. Path. Bact.*, 37, 473.

- Orksov, H. and Christensen, N.J. (1966) Disappearance rate of exogenous human insulin. *Lancet*, 2, 701.
- Ostrander, L.D., Francis, T., Hayner, N.S. et al. (1965) The relationship of cardiovascular disease to hyperglycaemia. *Ann. int. Med.*, 62, 1188.
- Paul, O., Lepper, M.H. and Phelan, W.M. (1963) A longitudinal study of coronary heart disease. *Circulation*, 28, 20.
- Pelkonen, R., Meittinen, T.A., Taskinen, M.R. et al. (1968) Effect of acute elevations of plasma glycerol triglyceride and FFA levels on glucose utilization and plasma insulin. *Diabetes*, 17, 76.
- Perley, M.J. and Kipnis, D.M. (1967) Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects. *J. clin. Invest.*, 46, 1954.
- Peters, N. and Hales, C.N. (1965) Plasma-insulin concentrations after myocardial infarction. *Lancet*, 1, 1144.
- Poffenbarger, P.L., Ensinnck, J.W., Hepp, D.K. et al. (1968) The nature of human serum insulin-like activity. *J. clin. Invest.*, 47, 301.
- Porte, D. (1967) A receptor mechanism for the inhibition of insulin release by epinephrine in man. *J. clin. Invest.*, 46, 86.
- Power, L., Lucas, C. and Conn, J.W. (1965) Augmentation of insulin-like activity. *Diabetes*, 14, 10.
- Prout, T.E., Odak, V. Rendrines, G.T. et al. (1963) The insulin carrying protein of normal human serum. *Diabetes*, 12, 144.
- Prout, T.E. (1963) Chemical structure of insulin in relation to biological activity and to autogenicity. *Metabolism*, 12, 673.
- Pruitt, K.M., Boshell, B.R. and Krersberg, R.A. (1966) Effect of pancreatic proteins on insulin assay systems. *Diabetes*, 15, 342.
- Pyke, D.A. and Taylor, K.W. (1967) Glucose tolerance and serum insulin in unaffected identical twins of diabetics. *Brit. med. J.*, 4, 21.
- Rabinowitz, D. and Zierler, K.L. (1962) Forearm metabolism in obesity and its response to intra-arterial insulin. *J. clin. Invest.*, 41, 2173.

- Rabinowitz, D. and Zierler, K.L. (1962) Role of free fatty acids in forearm metabolism in man, quantitated by use of insulin. *J. clin. Invest.*, 41, 2191.
- Rabinowitz, D., Merrimee, T.J., Maffezzoli, R. et al. (1966) Patterns of hormonal release after glucose, protein, and glucose plus protein. *Lancet*, 2, 454.
- Rafaelson, O.J., Lauris, V. and Renold, A.E. (1965) Localized intraperitoneal action of insulin on rat diaphragm and epididymal adipose tissue in vivo. *Diabetes*, 14, 19.
- Randle, P. (1954) Plasma and insulin activity in acromegaly assayed by the rat diaphragm method. *Lancet*, 1, 441.
- Randle, P., Garland, P., Hales, C. et al. (1963) The glucose fatty acid cycle. *Lancet*, 1, 785.
- Rapaport, M.I. and Hurd, H.F. (1964) Thiazide-induced glucose tolerance treated with potassium. *Arch. int. Med.*, 113, 405.
- Rasio, E.A., Soeldner, J.S. and Cahill, G.F. (1965) Insulin and insulin-like activity in serum and extravascular fluid. *Diabetologia*, 1, 125.
- Rasio, E.H., Soeldner, J.S. and Cahill, G.F. (1967) Diffusion of glucose, insulin, inulin and Evans blue protein into thoracic duct lymph of man. *J. clin. Invest.*, 46, 903.
- Rasio, E.H., Hill, G.J., Soeldner, J.S. et al. (1967) Effect of pancreatectomy in the dog. *Diabetes*, 16, 551.
- Reaven, G.M. and Miller, R. (1968) Study of the relationship between glucose and insulin responses to an oral glucose load in man. *Diabetes*, 17, 560.
- Reaven, G.M., Lerner, R.L., Stern, M.P. et al. (1967) Role of insulin in endogenous hypertirglyceridemia. *J. clin. Invest.*, 46, 1756.
- Remein, Q.R. and Wilkerson, H.L. (1961) The efficiency of screening tests for diabetes. *J. chron. Dis.*, 13, 6.
- Reiser, P. and Reiser, C.H. (1964) Anabolic response of diaphragm muscle to insulin and to other pancreatic proteins. *Proc. Soc. exp. Biol. Med.*, 116, 669.
- Renold, A.E., Martin, D., Magenais, Y. et al. (1960) Measurement of small quantities of insulin-like activity using rat adipose tissue. *J. clin. Invest.*, 39, 1487.

- Roberts, W. (1964) Alimentary hyperglycaemia simulating diabetic ketoacidosis. *Lancet*, 2, 754.
- Redbard, D., Rayford, P.L., Cooper, J.A. et al. (1968) Statistical quality control of radioimmunoassays. *J. clin. Endocr.*, 28, 1412.
- Rose, G.A. and Blackburn, H. (1966-1968) Cardiovascular survey methods. World Health Organization, Geneva.
- Roth, J., Glick, S., Yalow, R. et al. (1964) The influence of blood glucose on the plasma concentration of growth hormone. *Diabetes*, 13, 355.
- Rubenstein, A.H. and Spitz, I. (1968) Role of the kidney in insulin metabolism and excretion. *Diabetes*, 17, 161.
- Rubenstein, A.H., Cho, S. and Steiner, D. (1968) Evidence for proinsulin in human urine and serum. *Lancet*, 1, 353.
- Ruderman, N.B., Toews, CVJ. and Shafrir, E. (1969) The role of FFA in glucose homeostasis. *Arch. int. Med.*, 123, 299.
- Sagild, V., Anderson, V. and Andreassen, P.B. (1961) Glucose tolerance and insulin responsiveness in experimental potassium depletion. *Acta med. scand.*, 169, 243.
- Salans, L.B. and Reaven, G.M. (1966) Effect of insulin pre-treatment on glucose and lipid metabolism of liver slices from normal rats. *Proc. Soc. exp. Biol. (N.Y.)*, 122, 1208.
- Salans, L.B., Knittle, J.L. and Hirsch, J. (1968) The role of adipose cell size and adipose tissue sensitivity in the carbohydrate intolerance of human obesity. *J. clin. Invest.*, 47, 153.
- Samaan, N. and Fraser, R. (1963) "Typical" and "atypical" serum insulin-like activity in untreated diabetes mellitus. *Lancet*, 2, 311.
- Samaan, N., Dollery, C.T. and Fraser, R. (1963) Diabetogenic action of benzothiadiazines. *Lancet*, 2, 1244.
- Samaan, N., Brown, J., Fraser, R. et al. (1965) Effect of obesity and of starvation on insulin activity. *Brit. med. J.*, 1, 1153.
- Samols, E. (1965) Immunochemical aspects of insulin. In *On the Nature and Treatment of Diabetes* Ed. B.S. Leibel and G.A. Wrenshall. p. 227. Excerpta Medicine Foundation, Amsterdam.

- Samols, E. and Bilkus, B. (1964) A comparison of insulin immunoassays. *Proc. Soc. exp. Biol. Med.*, 115, 79.
- Samols, E. and Marks, V. (1964) Insulin assay in insulinomas. *Brit. med. J.*, 1, 507.
- Samols, E. and Marks, V. (1966) Disappearance rate of endogenous insulin in man. *Lancet*, 2, 700.
- Samols, E. and Ryder, J.A. (1961) Studies on tissue uptake of insulin in man using a differential immunoassay for endogenous and exogenous insulin. *J. clin. Invest.*, 40, 2092.
- Samols, E., Tyler, J., Mani, M. et al. (1965) Stimulation of glucagon secretion by oral glucose. *Lancet*, 2, 1257.
- Sanger, F. (1955) La structure de l'insuline. *Bull. Soc. Chim. Biol.*, 37, 23.
- Schalch, D.S. (1967) The influence of physical stress and exercise on growth hormone and insulin secretion in man. *J. Lab. clin. Med.*, 69, 256.
- Schuster, T.M. (1966) Possible structural similarity between insulin and glucagon. *Nature*, 209, 302.
- Scott, D.A. (1934) Crystalline insulin. *Biochem. J.*, 28, 1592.
- Seltzer, H.S. and Harris, V.L. (1964) Exhaustion of insulogenic reserve in maturity-onset diabetic patients during prolonged and continuous hyperglycaemic stress. *Diabetes*, 13, 6.
- Seltzer, H.S. and Smith, W.L. (1959) Plasma insulin activity after glucose: an index of insulogenic reserve in normal and diabetic man. *Diabetes*, 8, 417.
- Seltzer, H.S., Fajans, S.S. and Conn, J.W. (1956) Spontaneous hypoglycaemia as an early manifestation of diabetes mellitus. *Diabetes*, 5, 437.
- Seltzer, H., Allen, E., Herron, J. et al. (1967) Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. *J. clin. Invest.*, 46, 323.
- Sheldon, J. and Taylor, K.W. (1966) The immunoassay of insulin in human serum treated with sodium EDTA. *J. Endocr.*, 33, 157.

- Sims, E.A. and Horton, E.S. (1968) Endocrine and metabolic adaptation to obesity and starvation. Amer. J. clin. Nutr., 21, 1455.
- Siperstein, M.D., Unger, R.H. and Madison, L.L. (1968) Studies of muscle capillary basement membranes in normal subjects, diabetic and prediabetic patients. J. clin. Invest., 47, 1973.
- Skom, J.H. and Talmage, D.W. (1958) Non-precipitating insulin antibodies. J. clin. Invest., 37, 783.
- Soeldner, J.S. and Slome, D. (1965) Critical variables in the radioimmunoassay of serum insulin using the double antibody technique. Diabetes, 14, 771.
- Soeldner, J.S., Gleason, R.E., Williams, R.F. et al. (1968) Diminished serum-insulin response to glucose in genetic prediabetic males with normal glucose tolerance. Diabetes, 17, 17.
- Solomon, S.S., Ensink, J.W. and Williams, R.H. (1968) Effect of starvation on plasma immuno-reactive insulin and NSILA in normal and obese humans. Metabolism, 17, 528.
- Somersalo, O. (1950) Staub effect in children. Acta Paediat., Suppl. 78.
- Sonksen, P.H., Ellis, J.P., Lowy, C. et al. (1965) Plasma insulin: a correlation between bioassay and immunoassay. Brit. med. J., 2, 209.
- Sowton, E. (1962) Cardiac infarction and the glucose tolerance test. Brit. med. J., 1, 84.
- Stadie, W.C., Hangaard, N. and Vaughan, M. (1952) Insulin binding with isotopically labelled insulin. J. Biol. Chem., 199, 729.
- Stamler, J., Pick, R. and Katz, L.N. (1960) Effect of insulin in the induction and regression of atherosclerosis in the chick. Circulation, Res., 8, 573.
- Stamler, J. (1965) Role of cholesterol in atherosclerosis. Acta cardiol., Suppl XI, 499.
- Steele, R., Bjerknes, C., Rathgeb, I. et al. (1968) Glucose uptake and production during the oral glucose tolerance test. Diabetes, 17, 415.
- Steiner, D.F., Hallund, O., Rubenstein, A. et al. (1968) Isolation and properties of proinsulin ... from crystalline bovine insulin. Diabetes, 17, 725.

- Steinke, J., Soeldner, J.S., Camerini-Davalos, R. et al. (1963) Studies on serum insulin-like activity in prediabetes and early overt diabetes. *Diabetes*, 12, 502.
- Stenhouse, N.S. (1965) Multiple regression analysis. University of Western Australia Computer Programme Library. UWA 6-06.0010.
- Stern, M.P., Farquhar, J.W., Silvers, A. et al. (1968) Insulin delivery rate into plasma in normal and diabetic subjects. *J. clin. Invest.*, 47, 1947.
- Stout, R.W. (1968) Insulin-stimulated lipogenesis in arterial tissue in relation to diabetes and atheroma. *Lancet*, 2, 702.
- Stout, R.W. (1969) Insulin stimulation of cholesterol synthesis by arterial tissue. *Lancet*, 2, 467.
- Streeten, D.H.P., Gerstein, M.M., Marmor, B.M. et al. (1965) Reduced glucose tolerance in elderly human subjects. *Diabetes*, 14, 579.
- Tanner, J.M. (1959) The measurement of body fat in man. *Proc. nutr. Soc.*, 18, 148.
- Taylor, K.W. and Randle, P.J. (1959) The effect of insulin antiserum on the insulin activity of serum and protein fractions of serum. *J. Endocr.*, 19, 221.
- Taylor, K.W., Sheldon, J., Pyke, D.A. et al. (1967) Glucose tolerance and serum insulin in the unaffected first-degree relatives of diabetes. *Brit. med J.*, 4, 1967.
- Tomasi, T., Sledz, D. and Wales, J.K. et al. (1967) Insulin half-life in normal and diabetic subjects. *Proc. Soc. exp. Biol. Med.*, 126, 315.
- Tomizawa, H.H. and Halsey, D. (1959) Isolation of an insulin-degrading enzyme from beef liver. *J. biol. Chem.*, 234, 307.
- Trayner, I.M., Welborn, T.A., Rubenstein, A.H. et al. (1967) Serum and urine insulin in late pregnancy and in a few pregnant latent diabetics. *J. Endocr.*, 37, 443.
- Tzagournis, M. and Skillman, T (1969) The mechanism of glucose intolerance after starvation. *Diabetes*, 18, (Supple. 1), 374.

- Tzagournis, M., Seidensticker, J.F. and Hamwi, G.J. (1967) Serum insulin, carbohydrate, and lipid abnormalities in patients with premature coronary heart disease. *Ann. int. Med.*, 67, 42.
- Tzagournis, M., Chiles, R., Ryan, J.M. et al. (1968) Interrelationships of hyperinsulinism and hypertriglyceridemia in young patients with coronary heart disease. *Circulation*, 38, 1156.
- Unger, R.H. and Eisentraut, A.M. (1969) Enter-insular axis. *Arch. int. Med.*, 123, 261.
- Unger, R.H., Eisentraut, A.M. and Madison, L.L. (1963) The effects of total starvation upon the levels of circulating glucagon and insulin in man. *J. clin. Invest.*, 42, 1031.
- Vallance-Owen, J. (1964) Synalbumin insulin antagonism. *Diabetes*, 13, 241.
- Vallance-Owen, J. (1966) The inheritance of essential diabetes mellitus from studies of the synalbumin insulin antagonist. *Diabetologia*, 2, 248.
- Vallance-Owen, J. and Ashton, W.L. (1963) Cardiac infarction and insulin antagonism. *Lancet*, 1, 1226.
- Vallance-Owen, J. and Hurlock, B. (1954) Estimation of plasma insulin by the rat diaphragm method. *Lancet*, 1, 68.
- Vallance-Owen, G. and Lilley, M.D. (1961) An insulin antagonist associated with plasma albumin. *Lancet*, 1, 804.
- Vallance-Owen, J. and Lilley, M.D. (1961) Insulin antagonism in the plasma of obese diabetics and pre-diabetics. *Lancet*, 1, 806.
- Vallance-Owen, J., Dennes, E. and Campbell, P.N. (1958) The nature of the insulin antagonist associated with plasma albumin. *Lancet*, 2, 696.
- Vallance-Owen, J., Hurlock, B. and Please, W. (1955) Plasma-insulin activity in diabetes mellitus, measured by the rat diaphragm method. *Lancet*, 2, 583.
- Vance, J.E., Buchanan, K.D. and Williams, R.H. (1968) Effect of starvation and refeeding on serum glucagon and insulin levels. *J. Lab. clin. Med.*, 72, 290.
- Waddell, W.R. and Field, R.A. (1960) Carbohydrate metabolism in atherosclerosis. *Metabolism*, 9, 800.

- Wasserman, P., Broh-Kahn, R.H. and Mirsky, I.A. (1940) The antigenic property of insulin. *J. Immunol.*, 38, 213.
- Watson, D. (1960) A simple method for the determination of serum cholesterol. *Clin. Chem. Acta*, 5, 637.
- Welborn, T.A., Breckenridge, A.H., Rubenstein, A.H. et al. (1966) Serum-insulin in essential hypertension and in peripheral vascular disease. *Lancet*, 1, 1336.
- Welborn, T.A., Cumpston, G.N., Cullen, K.J. et al. (1969) The prevalence of coronary heart disease and associated factors in an Australian rural community. *Amer. J. Epidemiol.*, 89, 521.
- Welborn, T.A., Curnow, D.H., Wearne, J.T. et al. (1968) Diabetes detected by blood-sugar measurement after a glucose load: report from the Busselton survey, 1966. *Med. J. Austr.*, 2, 778.
- Welborn, T.A., Stenhouse, N.S. and Johnstone, C.G. (1969) Factors determining serum-insulin response in a population sample. *Diabetologia*, 5, 263.
- Weller, J.M. and Borondy, P.E. (1965) Effects of benzothiadiazine drugs on carbohydrate metabolism. *Metabolism*, 14, 708.
- Whyte, H.M. (1958) Body fat and blood pressure of natives in New Guinea: reflections on essential hypertension. *Austr. Ann. Med.*, 7, 36.
- Williamson, D.H., Mellanby, J. and Krebs, H.A. (1962) Enzymic determination of B hydroxybutyric acid and acetoacetic acid in blood. *Biochem. J.*, 82, 90.
- World Health Organization. (1959) The fourth international standard for insulin. *Bull. WHO*, 20, 1209.
- Wrenshall, G.A., Bogoch, A. and Ritchie, R.C. (1952) Extractible insulin of pancreas. *Diabetes*, 1, 87.
- Wright, A.D., Welborn, T.A. and Fraser, T.R. (1968) Effect of human growth hormone on "insulin basic protein complex" *Nature*, 210, 418.
- Wright, P.M. (1961) Properties of insulin antibodies produced by the guinea-pig, horse, sheep and man. *Diabetes*, 11, 519.

- Yalow, R.S. and Berson, S.A. (1960) Immunoassay of endogenous plasma insulin in man. *J. clin. Invest.*, 39, 1157.
- Yalow, R.S. and Berson, S.A. (1960) Plasma insulin concentrations in non-diabetic and early diabetic subjects. *Diabetes*, 9, 254.
- Yalow, R.S. and Berson, S.A. (1961) Immunoassay of insulin content of crystalline glucagon. *Proc. Soc. exp. Biol. Med.*, 107, 148.
- Yalow, R.S. and Berson, S.A. (1964) Immunoassay of plasma insulin. *Meth. Biochem. Anal.*, 12, 69.
- Yalow, R.S., Black, H., Villazon, M. et al., (1960) Comparison of plasma insulin levels following administration of tolbutamide and glucose. *Diabetes*, 9, 356.
- Yalow, R.S., Glick, S.M., Roth, J. et al. (1965) Plasma insulin and growth hormone levels in obesity and diabetes. *Ann. N.Y. Acad. Sci.*, 131, 357.
- Yalow, R.S., Goldsmith, S.J. and Berson, S.A. (1969) Influence of physiologic fluctuations in plasma growth hormone on glucose tolerance. *Diabetes*, 18, 402.
- Young, D.A.B. (1967) A serum inhibitor of insulin action on muscle II. *Diabetologia*, 3, 287.
- Young, D.A.B. and Benson, J. (1968) A serum inhibitor of insulin action on muscle II. *Diabetologia*, 4, 257.
- Young, D. and Lewis, A.H. (1947) Detection of hypoglycaemic reactions in the mouse assay for insulin. *Science*, 105, 368.
- Young, F.G. (1964) Insulin and insulin antagonism. *Endocrinology*, 73, 654.