Adrenergic Regulation of Insulin
Secretion in the Cold Acclimated Rat

By

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To

My parents, their grandchildren and my wife

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SUMMARY

This investigation was based on the hypothesis that long term cold exposure (cold-acclimation) reduces the overall amounts of insulin secreted by the pancreatic islets of Langerhans of the rat and possibly also reduces insulin synthesis. Evidence was obtained indicating that this effect was mediated through the sympathetic postganglionic terminals at the pancreatic beta-cells. The study was made on isolated perfused pancreases in a purpose-built perfusion system, and an artificial perfusion medium with a fluorocarbon as oxygen carrier were used.

It was confirmed that insulin secretion from the isolated perfused pancreas, in response to glucose stimulation follows a biphasic secretion pattern. Cold-acclimation reduces insulin secretion as a result of alpha-adrenergic activity. This effect was reversed by the action of the alpha-adrenergic antagonist phentolamine. A similar inhibitory effect on insulin release was achieved by perfusion with noradrenaline (40 ng/100 ml), although not to the same extent of inhibition which prevailed during cold-acclimation. The results suggested that the demonstrated alpha-adrenergic inhibition is directly effected by the pancreatic sympathetic fibres rather than by adrenal medullary secretion of catecholamines. Insulin output was measured by radioimmunoassay and the results were statistically analysed and discussed. It was concluded that during cold-acclimation, inhibition of insulin secretion and enhancement of catecholamine secretion enabled the cold-acclimated rat to maintain an adequate supply of thermogenic substrates for peripheral metabolism.

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INTRODUCTION

I.   Preamble

This introduction presents and discusses evidence concerning the dynamics of glucose-induced insulin secretion as well as other factors influencing insulin release from the beta-cells of the islets of Langerhans, especially the catecholamines and the autonomic nervous system. Both short- and long-term cold exposures, are unique in their direct and indirect stimulatory effects on metabolism. Cold exposure has been shown to enhance the activity of the sympathetic nervous system and consequently to increase levels of catecholamine secretion.

The objectives of the present investigation are to study the secretory dynamics of the isolated perfused pancreas of the cold-acclimated rat, in relation to catecholamine and glucose sensitivity and to verify the source of the effective catecholamines. Thus, the principal hypothesis put forward will be that long-term cold exposure (cold-acclimation) reduces the overall amounts of insulin released and possibly in addition, reduces the level of insulin synthesis, and that this effect is due to the increased noradrenaline secretion of the adrenergic nerve endings in the vicinity of the beta-cells of the pancreas.

For this investigation a perfusion system was built in order to permit continuous monitoring of relavent physiological parameters. Insulin production was measured by a radio-immunological method.

It is hoped that the results will yield a better under-
standing of the regulation of thermogenic metabolism in long-term
cold.

II.   Insulin secretion and glucose

Increased local glucose concentration is one of the major
physiological stimuli for insulin secretion from the pancreatic
beta-cells. Many investigators in this field have demonstrated
that changes of glucose concentrations, both in vivo and in vitro,
induce parallel changes in insulin release from the pancreatic
islet tissue (Coore and Randle, 1964b; Grodsky and Bennett, 1963;
Malaisse, Malaisse-Lagae, and Wright, 1967a; Mangay, DeRuyter,
Tonber, Croughs, Schopman and Lenquin, 1967; Metz, 1960; Sussman,
Vaughan and Timmer, 1966). In the isolated perfused pancreas,
sudden increases of glucose concentration are followed by an
immediate release of insulin (Curry, Bennett and Grodsky, 1968a,b;
Grodsky, Batts, Bennett, Vcella, McWilliams and Smith, 1963;
Sussman et al., 1966). Detailed analysis of the actions of hypo-
and hyper-glycaemia have been performed on the isolated and per-
fused rat pancreas, after exclusion of all extrapancreatic tissue
(stomach, spleen, duodenum and jejunum) by the technique described
by Sussman et al. (1966) and Loubatières, Mariani and Chapal (1970 a,b).
Such a preparation permitted a conclusive exploration of the dynamics
of the secretion of insulin not afforded by other isolated pancreatic
tissue preparations such as isolated islets, slices or tissue fragments;
with this approach, the following facts have been established:

1.   In the absence of glucose in the perfusion
medium, the isolated perfused rat pancreas secretes
insulin at a basal rate of less than 2 ng/min. The
reported work does not clarify the osmolar effects
of the absence of glucose.

2.   In the presence of increasing concentrations
of glucose, the insulin secretion pattern of the
pancreas is markedly different.

It was shown that if the isolated and perfused
rat pancreas is submitted to continuous perfusion by
a physiological medium containing 17 mmol/l glucose,
there appears an immediate acute intense phase of
insulin secretion lasting approximately five to six
minutes. If the perfusion of glucose continues, a
second phase of insulin secretion follows, rising to a
plateau that is directly related to the concentration
of glucose. The relationship established between the
concentration of glucose in the medium and the quantity
of insulin secreted shows that in response to the change
of glucose concentration from 4.44 mmol/l to 16.65 mmol/l,
the corresponding change in the secretion rate of insulin
is from 4 ng/min. to about 200 ng/min.

3.   When glucose is removed from the perfusion medium,
the insulin secretion rate falls and rapidly attains the
level of basal secretion, even after glucose has been
continuously supplied for sixty minutes. This suggests

that glucose or its metabolites have a stimulating
effect of very short duration on insulin secretion
and that these stimulating substances are probably
rapidly destroyed, metabolised or removed.

Thus, it is well established that glucose
stimulates insulin release from the pancreas. This
raises the important question of whether glucose also
stimulates insulin biosynthesis and if so, by what
mechanism and whether such a mechanism has any effect
on the process of insulin release. Current investi-
gations centre on the hypothesis that some direct or
indirect product of glucose metabolism provides the
stimulus (Mayhew, Wright and Ashmore, 1969). Indeed,
investigators have shown a three to four fold increase
in the rate of incorporation of radioactive amino acids
into insulin following the perfusion of glucose in the
concentration range of 3-28 mmol/l (Clark and Steiner,
1969; Howell and Taylor, 1966; Lin and Haist, 1969;
Morris and Korner, 1970; Steiner, Cunningham, Spigelman
and Aten, 1967; Tanese, Lazarus, Devrim and Recant, 1970).
(The degree of stimulation of radioactive amino acid
incorporation into insulin ranged between three- and four-
fold in the different experiments). When islets are
stimulated maximally by glucose, insulin makes up as much
as fifty per cent or more of the total protein synthesized
(Steiner et al., 1967). Mannose stimulates insulin

biosynthesis, but less strongly than glucose, whereas
fructose, ribose and xylitol have no effect (Lin and
Haist, 1969). Mannoheptulose, an inhibitor of glucose
phosphorylation and of insulin secretion (Ashcroft and
Randle, 1968; Coore and Randle, 1964a), also inhibits
insulin biosynthesis (Lin and Haist, 1969). Pyruvate,
a readily oxidized substrate, does not stimulate
insulin biosynthesis, nor does it overcome the inhibitory
effects of mannoheptulose, indicating that increased
availability of energy is not the basis of the biosynthetic
stimulus of glucose (Lin and Haist, 1969). However, other
observations indicate that insulin secretion and synthesis
involve oxidative metabolism of glucose and energy
production. Glucose-stimulation is blocked by anoxia,
potassium cyanide and 2,4-dinitrophenol (Coore and Randle,
1964b; Malaisse, Malaisse- Lagae , and Wright, 1967a),
which implicates the necessity of oxidative processes. In
view of the metabolic blocking action of 2-deoxyglucose in
the utilization of glucose-6-phosphate it is suggested
that the glucose-stimulated insulin release occurs after
the formation and further metabolism of glucose-6-phosphate
(Kilo, Devrim, Baily and Recant, 1967). Whether receptors
for glucose or its various metabolites exist to account for
the stimulus to both biosynthesis and secretion of insulin
remains to be determined.

Various lipid metabolites are claimed to stimulate insulin secretion. However, in many cases their insulinotropic effect is relatively modest and is only apparent under favourable experimental conditions. Short-chain fatty acids (octanoate, valerate, butyrate, propionate, but not acetate) exhibit a mild stimulatory effect on insulin release in rat, rabbit and pig (Horino, Machlin, Hertelendry & Kipnis, 1968; Malaisse and Malaisse-Lagae, 1968). Thus, these acids have been shown to enhance glucose-induced insulin secretion from pancreatic tissue or isolated islets from fetal and adult rats in vitro (Gagliardino and Martin, 1966; Montague and Taylor, 1968). Whether lipid metabolites play a significant role in the regulation of insulin secretion under physiological conditions remains to be ascertained. Nevertheless, it has been proposed that their stimulation of insulin secretion could function during starvation in the control and maintenance of a low but vital concentration of plasma insulin which is necessary to prevent the development of fatal ketoacidosis (Madison, Mebane, Unger and Lockner, 1964; Seyffert and Madison, 1967). A similar vital role could prevail during cold exposure which shows a marked mobilization of lipid metabolites.

III. Cold-acclimation

Energy metabolism and associated phenomena during exposure to cold have been the subject of considerable research. Short term exposure to cold results in an immediate and apparent shivering thermogenesis, while long term exposure culminates in an altered steady state of non-shivering thermogenesis affected by corresponding physiological changes. The term "cold-acclimation" is restricted

to designate the effects of prolonged exposure to chronic cold, where this is the only factor being changed, whereas "cold-acclimatization" is used to describe seasonal changes in which multiple environmental effects are involved, i.e. where temperature is not the only variable factor (Hart, 1960, 1962).

The development of cold-acclimation requires a period of time usually estimated to be from two to six weeks by many observers (Chin & Cheng, 1976; Chowers, Siegel, Conforti & Baranes, 1977; Depocas, 1959a, 1959b, 1960b; Guernsey and Stevens, 1977; Himms-Hagen, 1972; Jansky, 1973; Kennedy, Hammond and Hamolsky, 1977; LeBlanc and Nadeau, 1961; Maekubo, Moriya and Hiroshige, 1977; Smith, 1962). During this time there is a gradual increase in cold resistance together with an increase in food consumption and an elevation of basal metabolic rate (Baker and Sellers, 1953). Peripheral and sometimes central temperature are also elevated and there is a decrease in shivering and an increase in non-shivering heat production with the associated physiological and biochemical changes (Foster, Frydman and Usher, 1977; Hart, 1960; Smith, 1962).

Depocas (1959a, 1962) and Depocas and Masironi (1960) reported a constant finding that the cold-acclimated rats had a higher concentration of plasma glucose, and a larger pool size of glucose than the normal or warm-acclimated rats. They also reported an increase in turnover rate of body glucose when liver glycogen reserves are depleted which implies that exposure to cold results in effective stimulation of gluconeogenesis.

This evidence militates against a preferential oxidation of fatty acids in the metabolic response to cold (suggested earlier by Kayser, 1937; and Page and Chenier, 1953).

In a more recent study, Himms-Hagen (1972) demonstrated that the white adipose tissue serves as a store of readily mobilized free fatty acid substrates for thermogenesis in other tissues during cold-acclimation. It has been shown that brown adipose tissue which is markedly hypertrophied during this period is the site for this thermogenic process (Dawkins and Hull, 1964; Hardman and Hull, 1970; Horwitz, 1971; Hull and Segal, 1965; Smith, 1962; Smith and Horwitz, 1969). Furthermore, Beck, Zaharko and Kalser (1967) reported that rats exposed for two to seven weeks to severe cold (5 $\pm$ 2°C) exhibited, in comparison with control rats, significant decreases in serum glucose. Similarly, Himms-Hagen (1972) reported that the principal, but not the only substrate utilized during cold exposure and cold-acclimation is lipid. The concentration of free fatty acid in the blood was observed to be higher than normal suggesting increased lipolysis and free fatty acid release. The cellular oxidation of plasma free fatty acid is increased as is their turnover rate and the store of triglycerides in the white adipose tissue decreases (Masironi and Depocas, 1961).

As mentioned earlier, the cold-acclimated animal undergoes various adaptive adjustments during this acclimation period in which the replacement of shivering thermogenesis by non-shivering thermogenesis occurs. There is considerable evidence that the

adaptation to non-shivering thermogenesis is associated with a

very marked enhancement of the thermogenic response to noradrenaline

and adrenaline (Depocas & Secours, 1960; Hannon, 1963; Himms-Hagen, 1972;

Hsieh and Carlson, 1957; Jansky, 1973). Euler (1971) reported that

during exposure to cold, a clear reaction of the animal is in many

cases a greatly increased concentration of noradrenaline in both

blood and urine. Similarly, Nathanielsz (1969) observed that cold

exposure is a very potent stimulus to the sympathetic nervous system

for there is an increase in catecholamine synthesis and secretion

which is mainly in the noradrenaline fraction. Thus, it is possible

that the changes induced by cold are attributed to reduced availability

of insulin in the white adipose tissue, to both reduced availability of

insulin and increased availability of noradrenaline in the heart and

to increased release of noradrenaline in brown adipose tissue

(Himms-Hagen, 1972). Baker and Ashworth (1958) showed that the

amount of insulin extractable from the pancreas of rats was decreased

by exposure to cold ($1.5^{o}C$) for a period of fifty days, at the same

time there was an increased sensitivity to insulin after cold-acclimation

which might have been the result of a decreased availability following

a reduced requirement for insulin. Perhaps the prolongation of this

reduced requirement then resulted in the reduction of the insulin

content of the pancreas. Beck et al. (1967), Howland (1967) and

Howland and Nowell (1968) demonstrated that rats exposed for two

to seven weeks to severe cold exhibited, in comparison with controls,

significant decreases in serum glucose and immunoassay insulin levels.

IV.   Catecholamines, the nervous system and insulin release

A remarkably wide variety of compounds can influence the secretion of insulin in intact animals. Compounds which stimulate its secretion include glucose (Ashcroft and Randle, 1968 a,b; Depocas, 1959 a,b; Grodsky, Curry, Bennett and Rodrigo, 1968; Kilo, Long, Bailey, Koch and Recant, 1962; Lerner, 1977; Nowell and Howland, 1966; Snyder, Kashket and O'Sullivan, 1970), free fatty acids (Horwitz, 1971; Seyfert and Madison, 1967), Ketone bodies (Balasse, Couturier and Frenckson, 1967; Goberna, Tamarit, Osorio, Fussganger, Tamarit & Pfeiffer, 1974; Madison et al., 1964; Malaisse and Malaisse-Lagae, 1968; Pi-Sunyer, Campbell and Hashim, 1970), amino acids (Basake, Lopez, Viktora and Wolff, 1971; Floyd, Fajans, Conn, Knopf and Rull, 1966; Floyd, Fajans, Pek, Thiffault, Knopf and Conn, 1970), intestinal hormones such as pancreozymin (Mahler and Weisberg, 1968), secretin (Deckert, 1968) and gastrin (Dupre, Curtis, Unger, Waddell, and Beck, 1969; Unger, Ketterer, Dupre and Eisentraut, 1967), and other hormones such as glucagon (Campbell and Rastogi, 1966; Deckert, 1968; Devrin and Recant, 1966; Karam, Grasso, Wegienka, Grodsky and Forsham, 1966; Ketterer, Eisentraut and Unger, 1967; Porte, 1967 b; Samols, Marri and Marks 1965, 1966), and adrenocorticotropic hormone (ACTH) (Sussman and Vaughan, 1967). That parasympathetic stimulation also increases insulin secretion is indicated by the atropine-sensitive stimulatory effect of adminstered parasympathomimetic compounds in vivo (Kajinuma, Kaneto, Kuzuya and Nakao, 1968) and in vitro (Malaisse, Malaisse-Lagae, Wright and Ashmore, 1967 b) and by the increase in insulin secretion produced by vagal stimulation both in vitro and in vivo (Bergman and Miller, 1973; Daniel and

Henderson, 1967; Frohman, Ezdinli and Javid, 1966, 1967; Kaneto,

Kosaka and Nakao, 1967; Kaneto, Kajinuma, Kosaka and Nakao, 1968).

The sensitivity of the insulinogenic response to these different

stimuli varies according to the species, which might be a purely

anatomical effect involving nerve terminals distribution.

Any effective regulatory influence of endogenous cate-

cholamines must be considered against this background of multi-

plicity of stimuli to insulin secretion. Activation of the

sympathetic nervous system would be expected not only to produce an

inhibition of insulin secretion but also to produce an increase in

the concentrations in blood of several metabolites such as glucose,

free fatty acids and ketone bodies, which may stimulate insulin

secretion. The physiological function of this inhibition of

insulin secretion becomes apparent when considered as prevention

of the enhancement of insulin secretion that would normally occur

in response to raised concentrations of these metabolites. This

inhibition by the catecholamines allows the pancreas to respond to the

increased concentrations of those metabolites in the blood due to

an ingestion of a meal in a different way from that when it responds

to the increased concentrations of the metabolites in the blood due

to the sympathetic-mediated stress reactions

In order to discuss the possibility of a role for sympathetic

tone in the basal regulation of insulin secretion, evidence for

which comes mainly from the use of blocking agents, it is necessary

first to consider briefly the effects of blocking agents on the

secretion of insulin in the intact animal. Infusion of adrenaline
or noradrenaline inhibits the rise in plasma insulin that would
otherwise occur in response to hyperglyceamia, produced either
by the catecholamines themselves or by adminstration of glucose
(Campbell and Rastogi, 1966; Hertelendy, Machlin, Gordon, Horino
and Kipnis, 1966; Karam et al., 1966; Kris, Miller, Wherry and Mason,
1966; Porte, Graber, Kuzuya and Williams, 1966; Porte and Robertson,
1973; Porte and Williams, 1966; Potter, Wilson and Ellis, 1977).
Alpha-adrenergic blockade with phentolamine prevents the inhibition
and permits a rise in plasma insulin concentration (Kansal and Buse,
1967; Porte, 1967 a); whereas Beta-adrenergic blockade (with
propanolol) potentiates the inhibition (Porte, 1967 b). The
reported result that alpha-adrenergic agonists inhibit insulin
release was made by many investigators (Buse, Allan, Kuperminc and
Buse, 1970; Cerasi, Effendic and Luft, 1969; Malaisse et al., 1967 b;
Yajima, Hosokawa and Ui, 1977).

Attempts to assess the extent to which catecholamines exert a
tonic inhibitory effect on insulin secretion under basal conditions
have employed the alpha-receptor blocking agent phentolamine.
Phentolamine does raise plasma insulin concentration (Bressler,
Cordon and Bredel, 1969; Frohman et al., 1967; Werrback, Gale,
Goodner and Conway, 1970), and potentiates the rise in insulin con-
centration produced by glucose (Buse et al., 1970; Cerasi et al.,
1969) or by theophylline (Turtle and Kipnis, 1967 b). This can
be interpreted as evidence for a continuous basal inhibition of

insulin secretion by the catecholamines. However, it should be
remembered that phentolamine also has other effects in intact
animals. It raises plasma free fatty acids concentration
(Boshart, Will, Pirre and Ringler, 1965) and raises plasma lactate
concentration by producing activation of muscle glycogenolysis
(Salvador, April and Lemberger, 1968); both effects apparently due
to increased catecholamine secretion, principally from the adrenal
medulla (Boshart et al., 1965; Salvador et al., 1968). Phento-
lanine also raises plasma corticosterone concentration in rats
(Govier, Lovenberg and Sjoerdsma, 1969). Thus, phentolamine
may not only prevent endogenous catecholamines inhibiting secretion
of insulin, but may at the same time cause the appearance in the
blood of free fatty acids which can stimulate insulin secretion.
That a hypoglycemia results (Salvador et al., 1968) is probably
due to the increased secretion of insulin. However, it is not clear
to what extent the increased insulin secretion can really be
attributed to reduction of sympathetic tone and to what extent it is
due to stimulation of insulin secretion by the raised free fatty
acids concentration. Unfortunately, the use of beta-receptor
blocking agents to prevent the rise in plasma free fatty acids
concentration would not be helpful in resolving this question because
these agents also block the rise in insulin secretion produced by a
variety of other stimuli, including the administration of phentolamine
(Bressler et al., 1969).

A great deal of evidence has now accumulated supporting
the hypothesis that both the sympathetic and the parasympathetic
nervous systems share the capacity to make 'time adjustments'to insulin
secretion rates. The islets of Langerhans are provided with a rich
sympathetic and parasympathetic innervation (Woods and Porte, 1974).
Frohman et al. (1966,1967) have shown that stimulation of the
vagus nerve in anaesthetized dogs is followed by an increase in
serum immunoreactive insulin. Vagotomy alone is accompanied by a
decrease in insulin level and no change in blood glucose.
Stimulation of the distal end of the sectioned vagus nerve leads to
a prompt rise in portal insulin and a small rise somewhat later in
peripheral plasma insulin; the rise in insulin was blocked by
atropine. Such studies of vagal stimulation and vagotomy, indirectly
indicate that cholinergic innervation of the islets may regulate
insulin release (Chowers, Lavy and Harpen, 1966; Daniel and
Henderson, 1967; Kaneto et al., 1967; Loubatières-Mariani, Chapal,
Alric and Loubatières, 1973; Miller and Whittenberger, 1968; Nelson,
Blachard, Cocchiara and Labat, 1967).

On the other hand, the demonstration that both adrenaline
(Loubatières et al. 1970 a; Porte et al., 1966) and noradrenaline
(Porte and Williams , 1966) inhibit the release of insulin from
the beta-cell which is induced by glucose, glucagon and tolbutamide
is clearly relevant since noradrenaline is the neurochemical
transmitter secreted at sympathetic nerve endings which are known
to be plentiful in the islets of Langerhans and possibly to

terminate at the beta-cells (Lacy, 1967; Woods and Porte, 1974).

Simultaneous intravenous infusion of a glucose load with nor-

adrenaline resulted in a lower immunoreactive insulin concen-

tration than expected during the infusion of noradrenaline, but

returned to normal (or higher) values after the noradrenaline

infusion was stopped (Porte and Williams, 1966).

Stimulation of the splanchnic nerve inhibits glucose-

induced insulin release (Bloom, Edwards and Vaughan, 1973; Kaneto,

Kajinuma, Hayashi and Kosaka, 1974). Although little is known of

the local concentration of noradrenaline at sympathetic effector

sites after stimulation of sympathetic nerves, conceivably these

concentrations are similar to the concentration of noradrenaline

in the plasma (Porte and Williams, 1966). Euler (1971) reported

that noradrenaline released from sympathetic nerve terminals is

known to appear in the blood. Thus, the level of noradrenaline in

the blood could therefore serve as an indicator of the rate of trans-

mitter release (Benedict, Fillenz, Stanford and Valero, 1977).

These observations support the hypothesis that the sympathetic

nervous system plays a tonic role (through the action of noradrenaline)

in the regulation of insulin release.

That this noradrenaline is from extra-adrenal origin is

shown in adrenalectomized rats which excreted as much noradrenaline

as intact animals on exposure to cold (Leduc, 1961 a,b; LeBlanc and

Nadeau, 1960) or more than the controls (Johnson, Schönbaum & Sellers,

1966). Moore, Calvert & Brody (1961) reported that after cold-

acclimation the increased content of catecholamines in the adrenal

medulla is not necessarily accompanied by increased secretion of

these amines. Furthermore, as far as the studies performed on
adrenalectomized human patients are concerned (Sacca, Perez,
Carteni and Rengo, 1977), it is important to mention that the
removal of the adrenal glands does not assure the elimination of
all catecholamine-producing chromaffin tissue deposits. An
increase in the urinary content of adrenaline has been observed
in many species after adrenal medullectomy in response to various
stimuli including insulin-induced hypoglycemia (Armin and Grant,
1959; DeBodo and Altszuler, 1958; Vendsalu, 1960), and cold
exposure (Leduc, 1961 a,b). In all these conditions it must be
assumed that catecholamines are derived from extra adrenal sources
which seem able to respond to the various stimuli. Although the
role of noradrenaline in the homeostatic response to insulin-induced
hypoglycemia is not fully understood, the aforementioned studies
strongly suggest the possibility that an excess of noradrenaline
in demedullated animals, particularly that released from post-
ganglionic neurons at the intra hepatic and intra pancreatic levels
may be of great importance in facilitating the re-establishment of
metabolic equilibrium following insulin hypoglycemia (Sacca et al.,
1977). These studies performed on adrenodemedullated rats provide
evidence that the integrity of the sympathetic nervous system is
essential for an efficient homeostatic response in situations such
as insulin-induced hypoglycemia or cold-exposure.

Catecholamines are thought to have a direct effect on the
secretion of insulin through their influence on the activity of the
beta-cell adenylcyclase (Cyclic AMP) system. It has been accepted

that beta-adrenergic stimulation of the beta-cell enhances insulin

secretion by activation of this system, raising the cyclic AMP

concentration within the cell and that alpha-adrenergic stimulation

inhibits insulin secretion by antagonizing this rise of the cyclic

AMP (Porte and Robertson, 1973). Adrenaline, in the presence of

theophylline, prevented the accumulation of cyclic AMP, but when

an alpha-adrenergic blocker (Phentolamine) was added, normal

accumulation of the nucleotide occurred; beta-adrenergic blockade

did not antagonise the action of adrenaline (Turtle and Kipnis,

1967 a). This suggests that production of cyclic AMP is reduced

to a low level when an alpha-adrenergic stimulation is applied.

Comparing these observations with the parallel effects upon insulin

secretion suggests that the cyclic AMP level in beta-cells can

modify the rate of insulin release.

That cyclic AMP has effects on ion flux, which in turn

affect insulin release, may be considered as another site of action

of the catecholamines on insulin secretion (Hales, 1970; Rasmussen

and Tenenhouse, 1968), whereby, it is suggested that the effects

of cyclic AMP may be mediated by an effect of the nucleotide to

increase the concentration of ionized calcium in cytoplasm; what-

ever their primary site of action, the inhibitory effect of cate-

cholamines is apparently mediated ultimately through a decreased

uptake of calcium ions by the beta-cells (Case and Clausen, 1973;

Malaisse, Brisson, Malaisse-Lagae, 1970; Malaisse, Malaisse-Lagae,

1970). Finally, the addition of adrenaline to an incubation medium

and the ommision of calcium ions (Grodsky & Bennett, 1966) from

the medium are the only two experimental conditions known to suppress, in vitro, the insulinotropic effect of all agents so far investigated.

V.   Insulin release rhythms

Plasma insulin levels in mammals are the end result of the effects of different mechanisms. Though plasma glucose levels play an important direct role, hormones and other factors also influence the pancreatic secretion of insulin. Considering the existence, in the rat, of a diurnal rhythm which might affect insulin secretion (Allen and Kendall, 1967), Jolin and Montes (1973) demonstrated that plasma insulin levels show higher values during daytime with a maximum at 1700 h. These spontaneous oscillations of plasma insulin levels are not merely a consequence of those of the glucose level. Circadian periodicity of ACTH (Clayton, Librik, Gardener & Guillmen, 1963), and glucocorticoids (Allen & Kendall, 1967) secretion levels have been reported in rats and these hormonal fluctuations could well affect the pancreatic secretion of insulin thereby modifying the levels of glucose and insulin in plasma. It is noteworthy to mention that the noradrenaline levels did not show any significant daily variations (Benedict et al., 1977 b). Thus, measurements of plasma insulin and glucose levels may give misleading results if control and experimental groups of animals are not examined at the same time of the day.

On the other hand, Howland and Nowell (1968) demonstrated seasonal changes of plasma insulin concentration in the rat; whereby, maximum concentrations were recorded in June and minimum concentrations in November. When the increase of plasma insulin following a glucose load was expressed as a percentage of the fasting plasma insulin concentration, it was found that the response to glucose loading, in terms of insulin secretion, was greater during the winter than the summer months. The rat, maintained in artificial cold however, displayed a very low response in January, concomitant with a high insulin sensitivity recorded at this time. The decrease of plasma insulin content, seasonally and in response to a low environmental temperature, is thought to be related to a proposed endogenous cycle of islet beta-cells activity and the winter-enhanced secretory capacity of the beta-cells was related to the increased turnover of glucose in the cold-exposed animal (Howland, 1967).

VI.   Perfusion techniques for the study of insulin secretion

Insulin secretion has been examined both in vivo and in vitro. Of the two basic procedures carried out in vitro, the first, introduced by Anderson and Long (1947), involves perfusion of the rat's isolated pancreas with diluted blood or synthetic medium; whereas the second, involves media of known composition in which pieces of pancreatic tissue, isolated islets or explants of cultured pancreatic tissue are incubated (Coore and Randle, 1964 b;

Hellerstrom, 1964; Malaisse et al., 1967-a; Malaisse, Malaisse-Lagae and Mayhew, 1967 c; Malaisse, Malaisse-Lagae, Lacy and Wright, 1967 d).

Two groups of investigators are mainly credited for recent work obtained with the perfused pancreas. Sussman et al. (1966) used pancreatic tissue completely separated from surrounding organs while Grodsky et al. (1963) and Loubatières, Mariani, Chapal and Portal (1967) left the stomach, spleen, and duodenum attached. Diluted blood or bicarbonate buffered medium was infused through the aorta or coeliac axis, and effluents were collected from the hepatic portal vein. These media were either recycled or allowed to collect after a single passage, substances under study being incorporated in recycled media or injected as a "pulse" for a single passage through the tissue. Insulin output from the per-fused pancreas was represented either in absolute units or in terms of its concentration in the effluent medium.

Although perfusates made up of whole or diluted blood have been considered to be optimally physiological, certain changes that occur in blood in a perfusion circuit result in a few dis-advantages such as:

1. plasma lipoproteins might be denatured at the fluid/air and fluid/glass interfaces. This results in the formation of fat emboli which increase vascular resistance (Belzer et al., 1968).

2.   glass and rubber surfaces stimulate platelet aggregation.

3.   the essential addition of anti-coagulants to blood, such as heparin, have some metabolic effects. Citrates and other chelating agents reduce the availability of certain important ions such as calcium and magnesium.

4.   haemolysis of red blood cells usually occurs in the perfusion apparatus and this is known to cause insulin destruction (Grodsky et al., 1963).

Nevertheless, blood contains all essential factors for proper organ function, and most of the listed disadvantages are being minimized by the use of better oxygenators, connecting tubing, and more suitable vasodilators.

In synthetic perfusion media the maintenance of colloid osmotic pressure is accomplished by the use of plasma substitutes or expanders such as albumin, gelatine or dextran which was used by Grodsky et al., (1963) for pancreas perfusion experiments. The simple exposition of Starling's law is that blood circulating through a capillary bed loses fluid to the interstitial space when the hydrostatic pressure within the capillary exceeds the sum of the external tissue pressure and the colloid osmotic pressure within the lumen of the vessel. Since the hydrostatic pressure drops between the arterial and venous ends of the capillary, fluid

is lost at the former end and then regained at the latter end
of the capillary. In perfusion experiments, the artifical medium
and perfusion pressure, as well as the venous pressure should be
selected to achieve a similar balance, so that net fluid loss from
the vascular compartment does not occur.

VII.   Fluorocarbons as oxygen carriers

It would seem preferable to produce a standardized synthetic
medium, which does not contain blood, that could be used in organ
perfusion.

Recently, oxygen-carrying fluorocarbon suspensions have
become available for use to overcome most problems associated with
the fragile red blood cell (Gollan and Clark, 1966; Sloviter and
Kamimoto, 1967).

Fluorocarbons are generally regarded as biologically inert
compounds (Clark and Gollan, 1966). They have high diffusion
coefficients for gases. A liquid fluorocarbon "FX-80", composed
predominantly of perfluorobutyl-tetrahydrofuran and its isomers
(3M Co.), has a relatively high solubility for oxygen and carbon-
dioxide and when saturated with oxygen, it contains 0.63 ml of the
dissolved gas per ml suspension (Sloviter and Kamimoto, 1967).
These workers also demonstrated that "FX-80" in a dispersed
colloidal suspension (by sonication) adequately carries out the
essential functions of the erythrocyte in perfusing the rat brain,
and recommended it for perfusion studies of other organs.

Geyer, Monroe and Taylor (1968) reported that certain fluoro-
carbons in a protein-free mixture can be formulated to function
well in place of blood in the living intact rat on a short term
basis and concluded that fluorocarbons and high-molecular weight
micellar substances hold promise for perfusion used both in vivo
and in vitro. On the other hand, Gollan, McDermott, Duggen and
Musil (1972) reported that fluorocarbon emulsions proved to be
inefficient in delivering oxygen to the tissues of intact animals.
The reason for this is probably due to the presence of protein
molecules in concentrations as low as 2 µg/100 ml, which change
interfacial tension at water-fluorocarbon interfaces (Rosenberg,
1971). In isolated organs the blood is usually washed out initially
with a large volume of perfusate, and the content of protein to be
selectively absorbed on the fluorocarbon microparticles is
probably too low to interfere with the delivery of a large volume
of dissolved oxygen to a small mass of tissue (Gollan et al., 1972).
Nevertheless, as other similar compounds become available, still
better preparations for such uses might be possible.

VIII. The oxygenator

An efficient reliable oxygenator is an essential require-
ment of an organ perfusion apparatus. The perfusion medium reaching
the organ should contain enough oxygen to fully supply the need of
the tissue under investigation.

There are various types of oxygenator. which vary as to their efficiency, adequacy and ease of use. These oxygenators used in perfusion of a wide range of organs have been fully described and reviewed by Norman (1968) and Ross (1972), including: (i) bubblers, (ii) falling columns, (iii) films, (iv) rotating chambers, (v) rotating discs or cylinders, (vi) membrane oxygenators.

The rotating cylinder oxygenator or screen oxygenator is generally used for small organ perfusions where haemoglobin-free media are used. A plastic-coated mesh cylinder revolves about its long axis within the chamber, carrying a film of the medium up into the gas filled compartment above. Excessive gas loss usually takes place in this oxygenator and the inflow oxygen tension may not be the same as that of the medium leaving it. Cleaning is difficult. This oxygenator has been successfully used in perfusion of the pancreas as employed by many investigators.

IX.   Assay of insulin

Initial attempts to assay insulin were based upon the biological activity of the hormone, a method which proved to be lacking in sensitivity. Yalow and Berson (1960) developed the radioimmunoassay procedure and a sensitive assay technique was found in the various modifications thereafter.

As summarized by Mayhew et al. (1969), a constant amount of anti-insulin serum is allowed to react with an excess of insulin, a constant trace amount of which is radioactively labelled with

^{131}I or ^{125}I. At the end of the period of incubation two forms

of insulin can be discerned: part of the hormone is bound by

antibodies in the anti-insulin serum, and the excess remains free

in solution; the two forms are termed "bound" and "free" insulin.

Several techniques have been used to separate and measure the

amounts of bound and free insulin in all such incubated mixtures

(Beck, Zaharko, Roberts, McNeil, King, and Blankenbaker, 1964;

Grodsky and Forsham, 1960; Hales and Randle, 1963; Herbert, Lau,

Bottleib & Bleicher, 1965; Karam, Grodsky & Forsham, 1963; Mitchell

and Byron, 1967; Morgan and Lazarow, 1963; Wright, Makulu, Malaisse,

Roberts & Yu, 1968; Zaharko and Beck, 1968).

From the relative amounts of radioactivity associated with the

free and bound fractions of insulin obtained with samples and with

standard solutions of insulin, the insulin contents of the samples

can be determined. Meaningful results should take into consideration

species differences concerning anti-insulin sera, standard insulin

references and unknown insulin samples.

MATERIALS AND METHODS

I. Experimental animals

Wistar albino rats (Rattus norvegicus) were used, because

most reported works on cold-acclimation and isolated pancreas

perfusion experiments were done on them, as well as the ease of

obtaining and maintaining these animals. The rats were all

males because they keep a less variable endocrine physiology

than females. The average age of the rats was three months at

the time they were sacrificed and they weighed between two and

three hundred grams. They were separated into two main categories:

1. The normal rats.

2. The cold-acclimated rats (which had been

continuously exposed to a low temperature ($4^{o}C$) for

a period of six weeks and thus had been fully

acclimated to cold (Chin and Cheng, 1976; Chowers et al.,

1977; Depocas, 1959 a,b; Guernsey and Stevens, 1977;

Himms-Hagen, 1972; Jansky, 1973; Kennedy et al., 1977;

Maekubo et al., 1977; Smith, 1962).

1. The normal rats

Forty two rats with average body weight of 245 g

were kept two in a cage (20 cm x 30 cm x 50 cm) in the

animal house, at room temperature $22^{o}C$, with a constant

twelve hour photoperiod from 0800 hours G.M.T. to 2000 hours

G.M.T.; they were given food (Spratt's Small Animals Diet

No. 1) and water ad libitum. They were fasted for twenty

hours before experiment. Twelve of these rats were
used to study the effect of glucose stimulation on
insulin release from the isolated-perfused pancreas.
This comprised the Main Control Set.

Thirty rats were used to establish a nor-
adrenaline dose-response effect on insulin release
by glucose stimulation from the isolated-perfused
pancreas. The different noradrenaline doses were
included in the perfusion media accordingly, and for
each dose six experimental perfusion replicates were
done. These comprised the noradrenaline control set.

2. The cold-acclimated rats

The cold-acclimated rats were used in four
different experimental sets as follows:

i. Six rats with average body weight 296.25 $\pm$ 1.54g,
were put three in a cage (30 cm x 40 cm x 70 cm) and
kept, in a constant temperature-room at $4^{\circ}C \pm 2^{\circ}$ with
the same photoperiod as the normal rats, for a period of
six weeks. They were given food and water ad libitum,
and were fasted for twenty hours before the experiment.
This set of rats was used to study the effect of glucose
stimulation on insulin release from the isolated-perfused
pancreas after cold-acclimation.

ii. Six rats with average body weight 208.83 $\pm$ 2.09 g,
were acclimated to the cold, as previously described,

and were used to study the effect of glucose

stimulation on insulin release from the isolated-

perfused pancreas in the presence of both nor-

adrenaline and phentolamine.

iii.   Six rats with average body weight 208.00 ± 1.63 g,

were acclimated to the cold, the same as the previous

sets except that on the fifth week of acclimation they

were   given   subcutaneous injections of phentolamine

(25 μg in 0.25 ml normal saline - Malaisse et al., 1967 b),

twice per twenty four hours at 0900 hours and 2100 hours

G.M.T., for a period of seven days. They were given food

and water ad libitum and were fasted for twenty hours

before experiment to   investigate . the effect of glucose

stimulation on insulin release from the isolated-perfused

pancreas after phentolamine treatment.

iv.   Six rats with average body weight 205.08 ± 1.48 g,

were acclimated to the cold, as previously described, but

on the fifth week of cold-acclimation they underwent

bilateral   adrenal-demedullation, after which they were

put back in the cold room to complete the six weeks period.

They were given food and water ad libitum and a normal

saline solution for drinking after the operation. They

were fasted for twenty hours before experiment which studied

the effect of glucose stimulation on insulin release from the

isolated-perfusion pancreas after a week of adrenal-demedullation.

II.   The pancreas perfusion apparatus

1.   General principles of isolated organ perfusion:

The successful development of techniques for
isolated organ perfusion is based on the realisation that
the maintenance of organ viability is essential both
in the period between cessation of blood flow through
the organ, and replacement of blood with an appropriate
perfusion medium, and also during the perfusion process,
where every effort must be made to maintain the organ
under physiological conditions.

Considerations in the design of a perfusion system
should come as close as possible to meet the following
criteria:

i.   The experimenter must have control over the
perfusion medium flow and perfusion pressure.

ii.   The concentration of the stimulatory substances
in the perfusing medium must be under experimental control.

iii.   The composition of the perfusing medium, apart
from the experimentally varied concentrations of stimu-
latory substances should be kept constant.

On those grounds, it is concluded that the only
certain methods of maintaining control over perfusate
composition would be to   prepare a large volume of
perfusate and pump it through the test organ in a single pass
making additions of metabolites or drugs as demanded by
the experimental schedule.

The advantages of the perfused pancreas preparations over other techniques used in vitro, such as isolated islets, slices or tissue fragments, are summarized by Loubatières, Mariani, Chapal and Portal (1967) as follows:

i.   Superficial and deep islets are equally provided with oxygen and nutrients, which are constantly replaced. Substrates and effectors arrive at the cell in the same physiologically normal way.

ii.   The islets remain in normal anatomical relationship with other cells and tissues of the organ, including blood vessels and intrinsic nerves.

iii.   The maintenance of the cellular integrity of the tissues can be confirmed at the end of an experiment by microscopic study.

iv.   There is the possibility of a control and an experimental period of study in the same pancreas.

2.   Construction:

The pancreas perfusion apparatus is shown in Fig. (1), and its diagramatic representation is in Fig. (2):

The main water bath (1) was made of perspex boards, one centimeter thick, and dimensions 25 cm x 25 cm x 40 cm. It was heated by the electric heating unit (2)

Figure 1.   The perfusion assembly.   (For further details
            of the main water bath and contents see Fig.1a,
            overleaf).
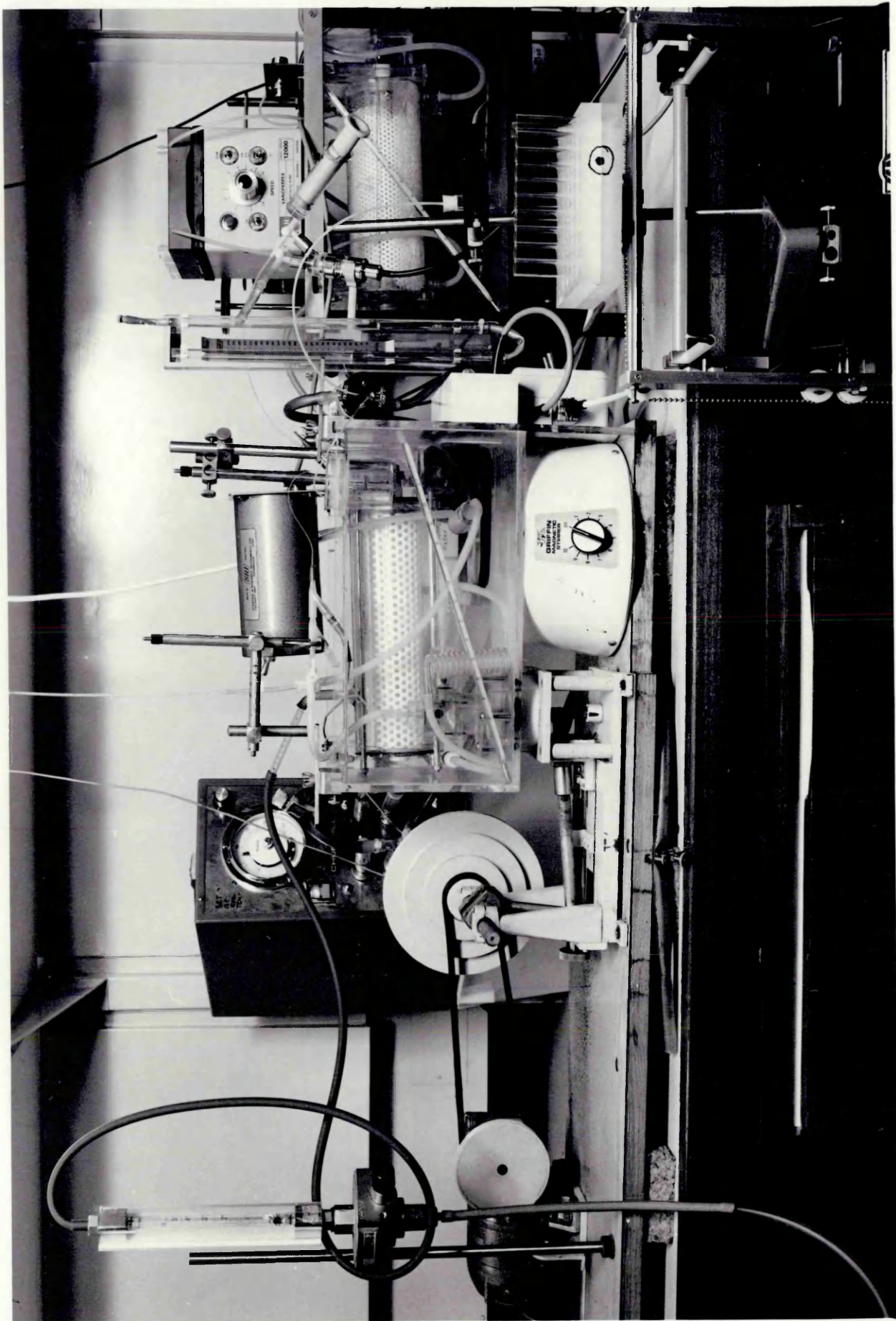
Figure 1a.   Detail of the main water bath of the perfusion
             assembly
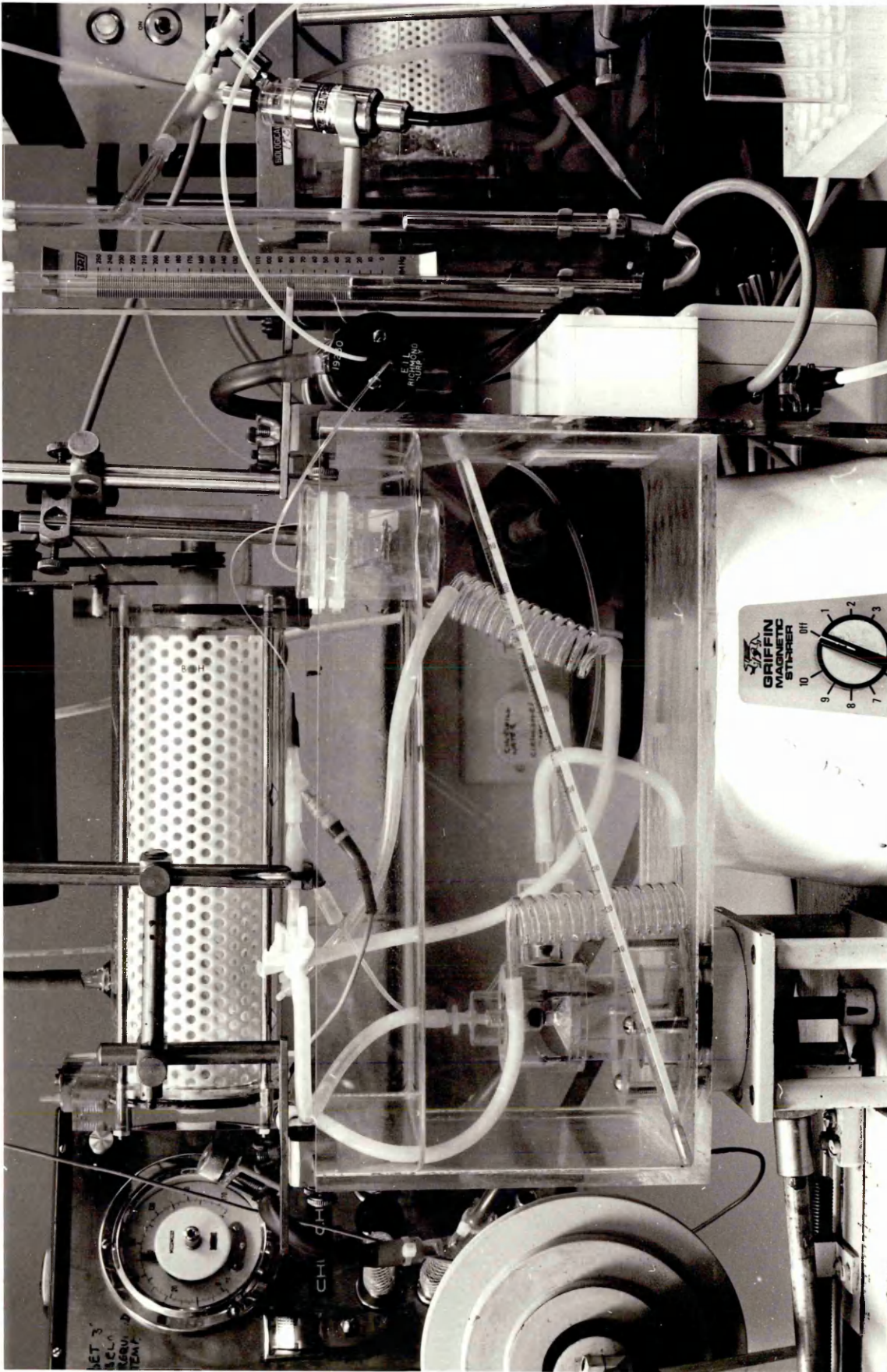
Key to numbers:

1.	Main water bath	12.	Drive belt	23.	Thermister
2.	Heating unit	13.	Peristaltic pump	24.	Luer-fitting tip of Y-tube
3.	Thermostat	14.	Glass-coil heat exchanger	24a.	Head of coeliac cannula
4.	Magnetic stirrer	15.	Bubble trap	25.	Perfusion chamber
5.	Oxygenator	16.	"	26.	Oxygen electrode
6.	"	17.	Three-way tap	27.	Drop counter
7.	Electric motor	18.	" "	28.	Fraction collector
8.	Glass-coil heat exchanger	19.	Millipore filter holder	29.	Electronic temperature meter
9.	Dale-Schuster pump	20.	Polythene Y-tube	30.	PO_2 meter
10.	Stroke-volume monitor	21.	Mercury manometer	31.	Drop recorder unit
11.	Electric motor	22.	Pressure transducer	32.	EMMA (multichannel amplifier) and ultra-violet recorder.
				33.	Mercury thermometer.
				34.	Collection tubes.

Figure 2.   Schematic diagram of the perfusion assembly.
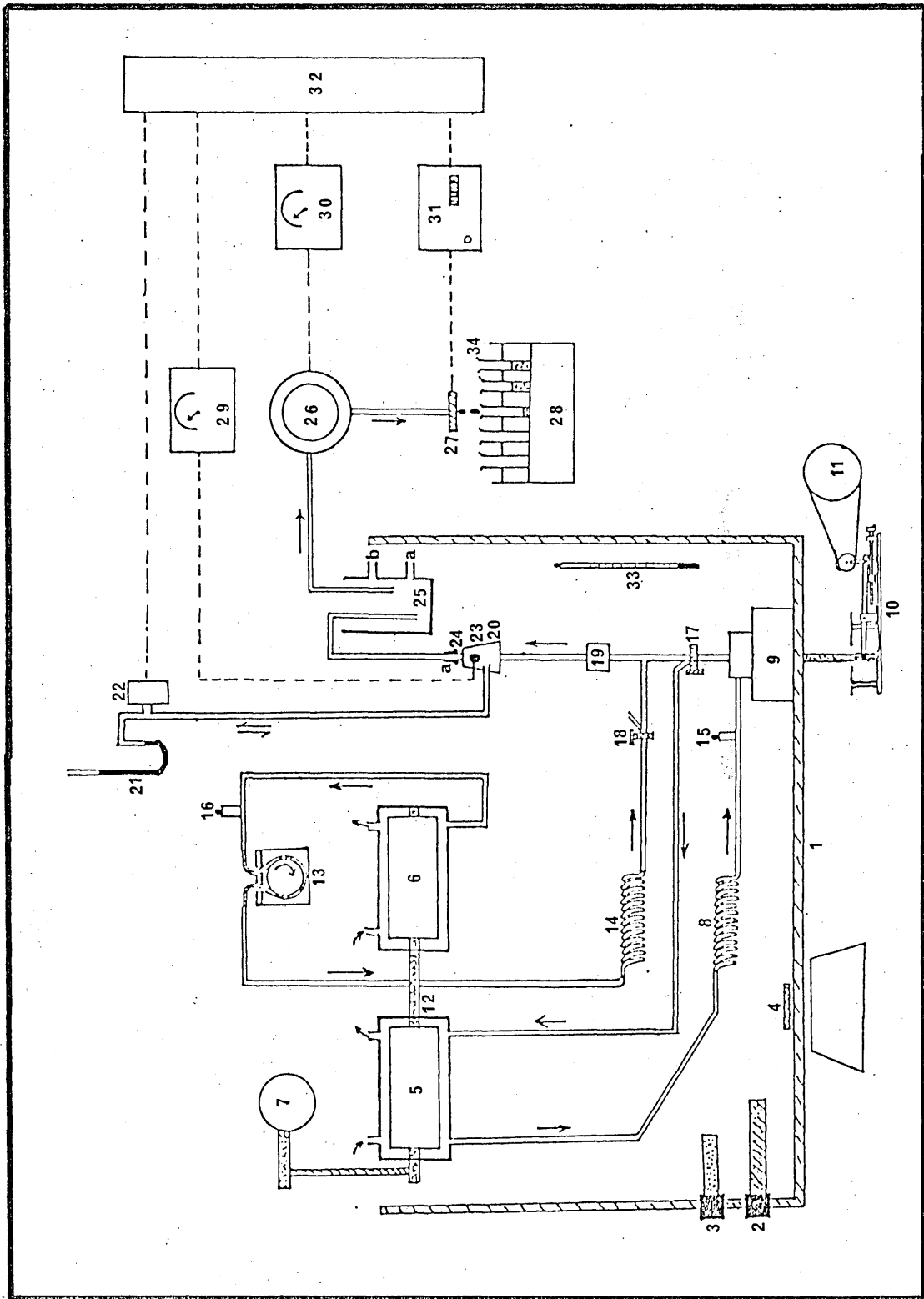
(220 volts, 700 Watts; SRI), and the temperature was maintained at a constant range (37.5°C ± 0.4°) by the thermostat (3) (Type T.S., N.C. ± 20°C, A.E.I. Ltd.). The uniformity of the bath temperature was ensured through continuous vigorous mixing of the water by the magnetic stirrer (4). Two rotating screen-cylinder oxygenators (5) and (6), contained separately in perspex jackets as perfusate reservoirs, were immersed in the bath (these rotating-screen cylinder oxygenators were a modification of that described by Ambeck, Beck Industries Inc., see Appendix 1). The first oxygenator (5) was run by an electric motor (7) (200-250 volts, S.R.I., Cat. No. 116). The cylinder revolved at fo rty revolutions per minute about its long axis. An outlet from this oxygenator's reservoir was connected by a silicon-rubber tube (internal diameter 2 mm) to a glass-coil heat exchanger (8) (internal diameter 3 mm; 12 turns, 2 mm apart; coil diameter 20 mm.) which lead to the pulsatile Dale-Schuster pump (9) (Dale and Schuster, 1928) with a variable stroke-volume monitor (10) and was driven by an electric motor (11). The pump ran at 145 strokes per minute. The second oxygenator (6) was connected through a drive belt (12) to the centre axis of oxygenator (5) and revolved the same; an outlet from its reservoir was connected to the peristaltic pump (13) (LKB, Varioperspex 12000, 240 volts) which was connected by a silicon-rubber tube (internal diameter 2 mm) to a glass-coil heat exchanger (14) which is the same as (8). Two bubble traps

(15) and (16) of 10 ml capacity were included in the

circuit immediately before pumps (9) and (13) res-

pectively. These bubble traps were used as safeguards

against possible inclusion of air bubbles in the

perfusates, although no frothing or foaming ever occurred

in the oxygenators. They were included before both

pumps in order to avoid dampening down the pulsatile

pressure gradients in front of the pumps. A Millipore

filter holder (19) (Swinex-25) contained a 0.22 μm

Millipore filter which removed any dust or large particle

inclusions from the perfusates.

At position (20) the pumped perfusates entered a

polythene Y-tube (internal diameter, 2 mm) which connected

the main flow line on one side to a mercury manometer (21)

and a Statham-type pressure transducer (22) (S.E.

Laboratories, S.E. 4-81, Mk 2); the other limb of the

Y-tube carried the main flow past the thermister (23)

(A solid glass bead N.T.C. Thermister Type U_{23}) which was

connected to an electronic temperature metre (29)

(Appendix 2). The perfusate flow continued into the main

perfusion luer-fitting tip (24) which accommodated the

coeliac cannula (24a) (Portex, Nylon Intravenous Cannula,

external diameter 0.63 mm, 2 FG, 30 cm long, Green Luer).

The glass perfusion chamber (25) (Pyrex, 50 ml beaker; with a lower inlet (25a) and an upper outlet (25b): which served to exchange a normal saline solution used to bathe the isolated-perfused pancreas) was partly immersed in the water bath.

An oxygen electrode (26) (a modification of the PO_2-electrode of a Blood Gas Analyser, Model 48C, EIL Ltd.) led into a drop counter (27) connected to the electronic drop recorder (31) (Appendix 3).

An automatic fraction collector (28) (Microcol T80, Gilson, Anachem Ltd.) was modified to be a moving-tube rack instead of a moving dropper. An Electro-Medical Multi-channel Amplifier (EMMA, SE4001, SE LABS, Ltd.) together with an ultra-violet recorder (SE 3006, SE LABS, Ltd.) (32) were used to record outputs from all of:

i.   The pressure transducer (22).

ii.   Temperature displayed on the electronic temperature meter (29).

iii.   Partial pressure of oxygen from meter (30) of Blood Gas Analyser.

iv.   Drop interval displayed on the electronic drop-recorder (31).

Two three-way taps (K-75a, Robineta 3 direction,

Dreiwegehahn) were included in the circuit, tap (17) on

the outlet from pump (9) and tap (18) immediately after

glass-coil heat exchanger (14). The functions of the

two taps will be described later. A mercury therm-

ometer (33) (-10°C to + 110°C) was used to check the

thermister-meter temperature readings. All connecting

tubes used in the circuit were silicon-rubber tubes of

internal diameter 2mm.

3.   <u>Operation of apparatus during perfusion</u>:

The system of pancreas perfusion used was a once-

through technique, i.e. the perfusates were not recycled

through the preparation.

With the main bath (1) Fig. (2), already at 37.5°C,

the two oxygenator reservoirs (5) and (6) were filled with

normal saline solution and rotation was commenced. The two

pumps (9) and (13) were put on, and the saline solution

was let to circulate throughout all the glass and silicone-

rubber tubes for thirty minutes, at the end of which the

saline was removed. 130 ml of the basal perfusion medium

(described later) were then put in the oxygenator reservoir

(6) and 130 ml of stimulation perfusion medium (described

later) were put in the oxygenator reservoir (5). These

volumes of media partly filled the reservoirs and immersed

the lower one third of the perforated screen-cylinders.

Oxygenation was started. The gas supply (O_2 : CO_2, 95% : 5%)

was delivered at a rate of 200 ml/min. by passing it
through a rotameter-type gas flow meter (Gaplaton Ltd.)
then was passed into a humidifier (a 250 ml flask,
containing 150 ml of normal saline), after which the
humidified gas mixture went into an inlet in the
oxygenator (5), from where it passed into the second
oxygenator (6) and finally vented through an outlet on
the second oxygenator. Pump (9) was started and tap (17)
was turned away from the main flow direction and was
connected through its third outlet by a silicon-rubber
tube (17a) back to oxygenator (5), thus keeping the
stimulation perfusion medium recirculating. Pump (13)
was started at the same time and tap (18) was kept opened
towards the main flow direction up to the luer-fitting
outlet (24) where the flow entered a connecting cannula
(Portex, Nylon Cannula, o.d. 2 mm, 80 cm long, yellow luer
at both ends) which was connected in turn back to the
oxygenator (6) and thus the basal perfusion medium was
kept recirculating. For thirty minutes, both perfusion
media were separately kept circulating by which time they
were equilibrated with the oxygen and carbon dioxide.

The coeliac axis cannulation was completed,
the connecting cannula was joined to the coeliac cannula,
and the basal perfusion medium started perfusing the pancreas.

Once the cannulation of the hepatic portal vein was completed and the perfusate out-flow started, the connecting cannula was removed altogether and the coeliac cannula (24a) was joined directly to the main perfusion outlet (24). The outlet of the hepatic portal cannula was connected to the oxygen electrode (26) from where the flow was directed into the drop counter (27) and then into the sample collection tubes (34) (polythene,0·5" x 3", round bottom tube, L,/072, Luckham Ltd.) in the automatic fraction collector (28).

Perfusion was kept running as such for twenty minutes in order to achieve complete stabilisation. Collection of samples then started, one tube every five minutes. The basal perfusion medium was kept perfusing the pancreas to collect the first five tubes, after which tap (18) was closed and tap (17) was opened to the main flow thus starting the perfusion with the stimulation perfusion medium. This permitted the collection of fifteen tubes, making a total of twenty tubes in one hundred minutes. Perfusion pressure was kept at 85 ± 10 mm Hg, depending on flow resistance of the preparation, to maintain an average outflow of 2.5 ml/5 min. Sample tubes were immediately removed and frozen at -10°C for later analysis. All sample collection was done between 1600 and 1800 hours G.M.T. (It was reported that the mean concentration of insulin in plasma measured at 1730 hours is very stable - Okajima and Ui, 1978).

A once-through normal saline solution at $37.5^{\circ}C$, and flow-rate of 6 ml/min. entered the perfusion chamber (25) at its lower inlet (25a) and was drained out from the upper outlet (25b); this provided a stable, warm and wet surrounding for the perfused pancreas and washed out pancreatic and other enzymes that might have been released in the chamber.

At the termination of each experiment, the perfused preparation was removed from the perfusion setup and a 10% methylene blue solution was injected through the coeliac cannula until it flowed out of the hepatic portal cannula. The pancreatic tissue stained with methylene blue was microdissected and removed, blot-dried by lightly pressing it between filter papers for five minutes and then was weighed. This was designated as the wet weight of the perfused pancreas.

4.   Cleaning the perfusion apparatus:

After each experiment the whole assembly was disassembled and all glassware, perspex, silicon-rubber tubing and cannulae were rinsed with tap water overnight and then washed with detergent and thoroughly rinsed with tap water, followed by distilled water and left to dry.

III.   Perfusion media

In general, a selected perfusion medium must meet certain requirements in order to achieve normal function of an organ in vitro; such a medium has to supply all the essential factors which the organ receives in vivo, taking special care not to introduce any harmful effectors. Thus, an optimum medium has to accomplish the following:

i.   a sufficient oxygen-carrying capacity

ii.   an adequately buffered physiological pH, usually 7.4, i.e. arterial pH.

iii. a physiological concentration of the principal ions (such as in Krebs-Henseleit bicarbonate medium (1932) as cited in Ross (1972).

iv.   a sufficient colloidal osmotic pressure, especially in the absence of blood, to balance the hydrostatic and tissue pressures.

The perfusion medium which was chosen for this investigation was Krebs-Henseleit bicarbonate medium (1932), which was described by Dawson, Elliott, Elliott and Jones (1969) and Ross (1972), and was used by many researchers with various alterations (Curry et al., 1968-a,b; Loubatieres et al., 1967; Sussman et al., 1966); it was made up of the following stock solutions:

1. NaCl $(0.154$ mol/l)   4. KH_2PO_4 $(0.154$ mol/l)

2. KCl $(0.154$ mol/l)   5. $MgSO_4 \cdot 7H_2O$ $(0.154$ mol/l)

3. $CaCl_2$ $(0.11$ mol/l)   6. $NaHCO_3$ $(0.154$ mol/l).

All salts were analytical quality (Analar, BDH). The sodium bicarbonate solution was bubbled with 100% CO_2 gas for one hour in order to convert any carbonate to bicarbonate ions, before preparing the following mixture: 100 parts (volume) of solution (1) were mixed with 4 parts of solution (2), 3 parts of solution (3), one part of solution (4), one part of solution (5) and 21 parts of solution (6). Thus, the final concentration of solutes in the solution were: 118.5 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4 \cdot 7H_2O$ and 24.9 mmol/l $NaHCO_3$. In case any turbidity of the solution was observed, it was reversed and cleared by bubbling a 5% CO_2 gas ($CO_2:O_2$, 5%:95%) for ten minutes.

To this solution the following were added:

a. Dextran   (Clinical grade, average Mol. Wt. 83300, Sigma Chemical Co.) at a final concentration of 4 gm/100 ml solution (Grodsky et al., 1963) for the maintenance of colloid osmotic pressure.

b. Adenosine   (Anhydrous, Mol. Wt. 267.2, Sigma Chemical Co.) at a final concentration of 1.5 mmol/l (Davis and Mepham, 1971, 1974), which maintains the integrity of the endothelial cells (Salzman, Chambers

and Neri, 1966) and acts as a

vasodilator of blood vessels

(Davis and Mepham, 1971, 1974;

Wolf and Berne, 1956).

c.   <u>Glucose</u>   (D (+) glucose-Dextrose, Mol. Wt.

180.16, Analar, Searle Co.) at the

following final concentrations:

1.   5.0 mmol/l in the basal perfusion medium,

which is considered as a weak non-stimulatory

concentration of glucose in rats (Loubatières-

Mariani <u>et al</u>., 1973; Malaisse <u>et al</u>., 1967 a,b).

2.   18.87 mmol/l in the stimulation perfusion

medium, which is a very potent stimulatory con-

centration of glucose in rats reported by many

investigators (Curry <u>et al</u>., 1968-a,b; Curry, 1971;

Grodsky <u>et al</u>., 1963; Loubatières-Mariani <u>et al</u>., 1973;

Malaisse <u>et al</u>., 1967 a,b).

Perfusate mixtures were filtered through a Whatman No. 1

filter paper in order to remove any dust or insoluble particles.

To two separate 26 ml volumes of basal perfusion medium and

stimulatory perfusion medium, 0.5 ml of FX-80 (perfluoro-2-butyl

tetrahydrofuran, ICN pharmaceuticals, Inc.) were added. Each volume

with the added FX-80 was subjected to ultrasonic disintegration

(Sloviter and Kamimoto, 1967) for two minutes with an MSE 150 Watts

(20 K Hz) disintegrator fitted with a 3.2 mm exponential probe.

The resulting suspension was homogenous and milky; and upon microscopic examination it showed uniform dispersal of FX-80 into droplets 6-9 μ in diameter, which was stable for two hours or more. These separate suspensions of FX-80 were returned to the basal and stimulatory perfusion media respectively, making the final volume of each 130 ml. No serum albumin was included in the perfusates because it was observed, in pilot experiments, that its absence did not affect the secretory response of the perfused pancreas, confirming the remark made by Joost, Beckmann, Holze, Lenzen, Poser and Hasselblatt (1976).

The basal and the stimulatory perfusion media were put separately in the two screen oxygenators, and both were oxygenated by a humidified gas mixture (O_2:CO_2, 95%:5%) for thirty minutes prior to the start of perfusion. Inflow and outflow pH of both media was the same (7.3), thus the buffer capacity was adequate. Both media, the basal and the stimulatory, were used in perfusing all the pancreases of this research; furthermore, in the five sets of experiments designed to establish a noradrenaline dose-response effect, the following noradrenaline (Levophed) concentrations, per 100 ml solution, were each simultaneously included in both media : 5 ng, 20 ng, 40 ng, 80 ng and 100 ng. It has been reported that the circulating noradrenaline level in normal rats is 25 $\pm$ 3 ng/100 ml (Grobecker, Sarrvedra, McCarty, Chiveh and Kopin, 1977), whereas Benedict et al., (1977 b) reported that the noradrenaline content of plasma in rats is 188 $\pm$ 8 ng/100 ml. Other relative works report the injection of 40 μg or 50 μg noradrenaline per 100 g intact rat (Holak, Baldys, Jarzab,

Wystrychowsky and Skrzypek, 1978; Kuroshima, Doi, Yahata and
Ohno, 1977). Finally, in the set of experiments performed to
study the simultaneous effects of noradrenaline and phento-
lamine, on the cold-acclimated rat pancreas, both the basal and
the stimulatory perfusion media were used with the addition of
100 ng noradrenaline and 50 µg phentolamine (Rogitine, CIBA)
per 100 ml of each of the media (Malaisse et al., 1967 b)

IV. Preparation and isolation of the pancreas for perfusion

To isolate and perfuse an organ, especially the rat pancreas,
is relatively a tedious technique. Nevertheless, the complexity
of such a perfusion is becoming reduced as simpler designs of
isolation procedures and perfusion apparatus become available.
Grodsky et al., (1963) introduced the technique of perfusing the
isolated pancreas in a block of tissue including all of the pancreas,
stomach, spleen and duodenum; this technique was adopted by many
investigators (Basake, et al., 1971; Loubatieres et al., 1967, 1973;
Penhos, Wu, Basake, Lopez and Wolff, 1969). Thus, the aim of the
preparation was to isolate in one physiological unit all of: the
pancreas, the duodenum, the spleen and the stomach, after proper
cannulation of the coeliac axis and the hepatic portal vein.

A rat, fasted for twenty hours, was weighed and then
anaesthetized by intraperitoneal injection with Nembutal (pento-
barbitone sodium, 60 mg Nembutal/ml, Abbot Labs. Ltd.) at a dose
of 5.5 mg/100 gm bodyweight, (Ross, 1972).

With the rat supine and its limbs extended, a mid-line
incision was made through the skin and the abdominal wall from
the pubis to the manubrium sterni, along the linea alba. The
cut abdominal wall was slightly retracted laterally. A cotton
wool pad dampened with warm normal saline was placed over the
abdominal organs to prevent drying. The dorsal aorta Fig. (3)
was located in the lumbar region posterior to the renal arteries
and was separated from the inferior vena cava and   connective
tissue for a short distance (approx. one centimeter) where a loose
ligature (1) Fig. (3) was placed. By displacing the intestine,
the stomach and the spleen to the right side of the animal, the dorsal
aorta was located anterior to the left renal artery where the superior
mesenteric artery and the coeliac axis branch. The area was cleaned of
fat tissue, and a loose ligature (2) was placed around the aorta between
the superior mesenteric artery and the coeliac axis. A third ligature
(3) was placed around the aorta anterior to the coeliac axis in the
vicinity of the crura of the diaphragm. The superior adrenal artery
was located and ligated by ligature (4). Three branches from the
coeliac axis: the left gastric artery, the right gastric artery and the
hepatic artery were located and ligated by three ligatures (5, 6, 7).
Blood vessels of the spleen were collected in three adjacent groups
and tied within three ligatures (8, 9, 10). Two loose ligatures (11)
and (12) were placed around the gastro-hepatic ligament.

At this stage, ligature (3) was firmly tied and, immediately,
a small incision was made in the wall of the aorta posterior to
ligature (1) and a cannula (Portex, Nylon Intravenous Cannula, external
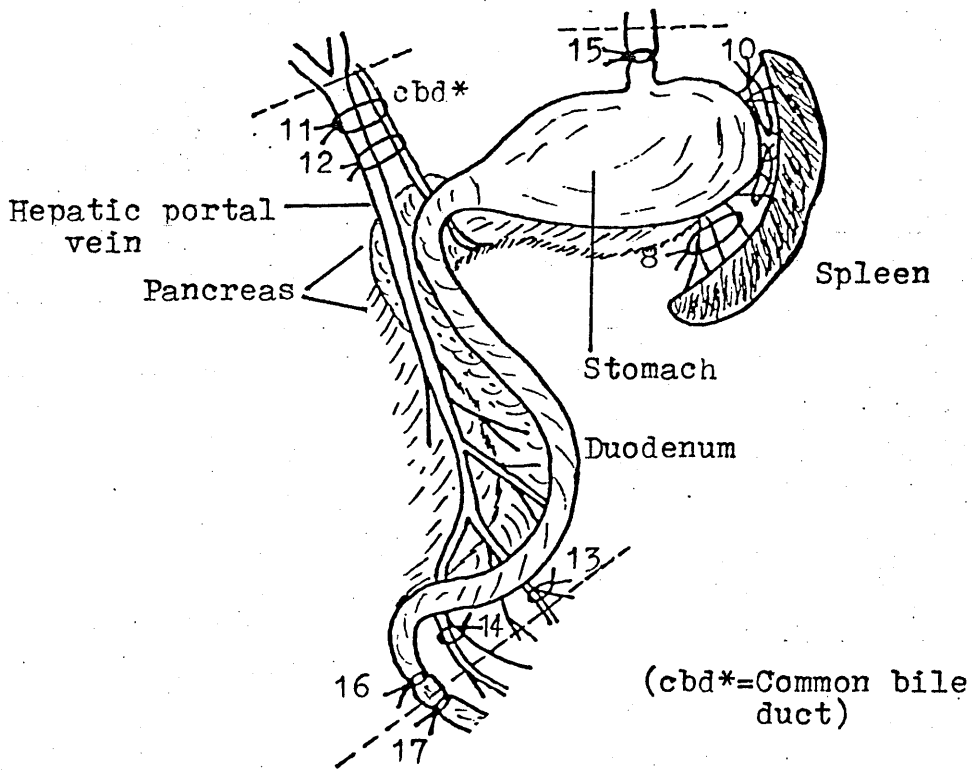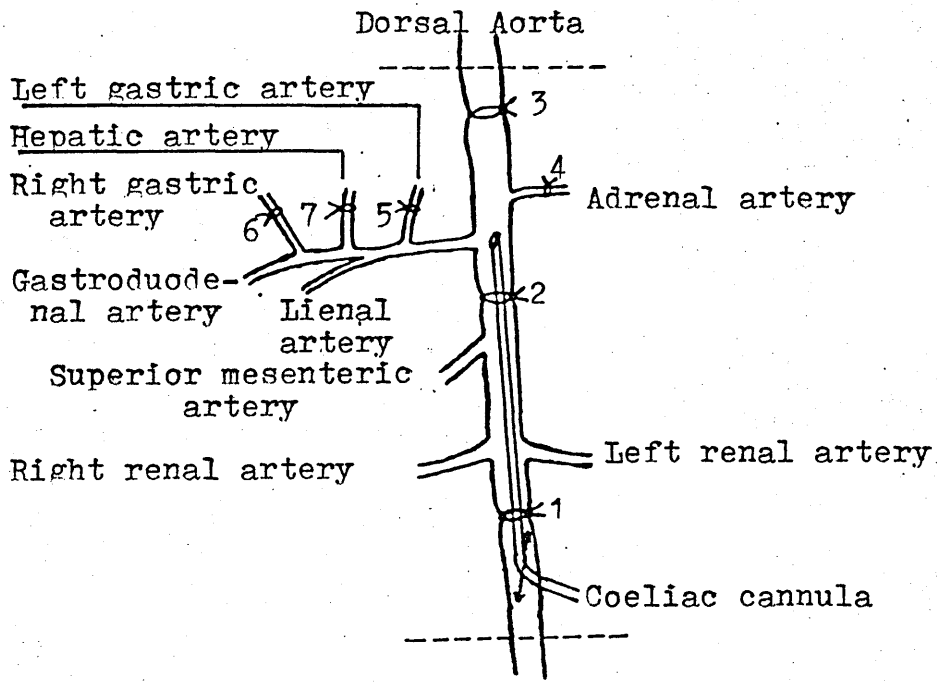
Figure 3. Schematic diagrams of the rat dorsal aorta and the
          major arteries (a), and the isolated perfused unit
          (b), for ligatures (1-17) refer to text. (----)
          indicates position of cuts.

diameter 0.63 mm, Luer mount), cut to 15 cm length, was introduced forward inside the aorta, till its tip reached the origin of the coeliac axis. Ligatures (1) and (2) were then tied and 0.1 ml (2 I.U.) heparin (Heparin, 1000 I.U. per ml; diluted 1:50 in normal saline) was introduced (Penhos et al., 1969) into the cannula, which was then connected to the main flow of the basal perfusate from the persfusion main output tip, by a thick cannula (Portex, Nylon Cannula, external diameter 2mm, 80 cm long, Luer connector at both ends).

A slit was made in the wall of the hepatic portal vein, between ligature (11) and the entry of the hepatic portal vein into the liver. Once it was ensured that a perfusate outflow was taking place from this slit, a 15 cm cannula (Portex, the same as the aortic cannula but no luer fitting) was introduced for a distance of about 5 mm inside the portal vein. The two ligatures (11) and (12) were tightened and tied. A slit was made in the wall of the common bile duct between ligature (12) and the pancreas in order to allow outflow of any exocrine secretion from the pancreas and prevent possible local oedema. Two ligatures (13) and (14) were placed around the major vessels leading into the hepatic portal vein from the parts of the intestines beyond the duodenum.

The oesophagus was ligated close to the stomach and cut distally (15). Two ligatures (16) and (17) were placed and tied around the junction of the duodenum and the jejunum, and a cut inbetween the two ligatures was made.

The aorta was separated posterior to ligature (1) and anterior to ligature (3), and the segment between these two points was freed of all dorsal attachments, thus the pancreas, the duodenum, the stomach, the spleen, the aortic segment with its coeliac cannula and the hepatic portal vein and its cannula were isolated in one block. The whole unit was washed twice with warm normal saline solution and transferred to the main perfusion chamber in the water bath. The coeliac cannula was connected directly to the main perfusion flow tip, after the removal of the thick connecting cannula. The entire operation took thirty to fourty minutes, with a maximum of two minutes anoxia during the coeliac axis cannulation.

The viability of the isolated perfused pancreas was assessed according to the following criteria:

1) The positive secretory response of the perfused pancreas to a glucose load.

2) Arterio-venous oxygen differences.

3) Glucose uptake by the perfused pancreas (and other tissues).

4) Increasing vascular resistance (a sign of failure of the perfusion - this usually happened after at least one hundred minutes of perfusion).

5) The physical appearance of the perfused pancreas and peristaltic movements of the duodenum.

ACQUISITION AND PROCESSING OF DATA

I.   Glucose analysis:

Determinations of the glucose concentrations
in both the basal and the stimulatory perfusion media were carried
out prior to perfusion in every experiment as well as all the
samples collected individually and in duplicates. The method used
was a commercial kit form of the enzymatic glucose oxidase technique
(GOD-PERID Method, The Boehringer Corporation, London, Ltd.), which
is summarized as follows: 0.1 ml (measured by Eppendorf Microlitre
Pipette, 100 μl) of unknown glucose solution was added to 1ml of
distilled water and 0.1 ml of this diluted solution was incubated
for thirty minutes at $37^{\circ}C$, with 5 ml of reagent (100 mmol/l
phosphate buffer pH = 7.0; 20 μg peroxidase (POD)/ml; 180 μg glucose
oxidase (GOD)/ml); 1.0 mg Azinoethyl-benthiazoline sulphonate
(PERID)/ml). Glucose is oxidised by the specific enzyme of glucose
oxidase (GOD) to gluconolactone which, in aqueous solution in the
presence of peroxidase (POD), is converted to gluconic acid:
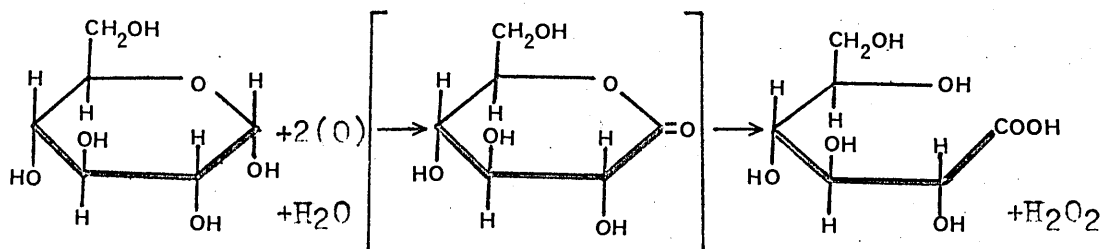
the hydrogen peroxide (H_2O_2) produced in the above reaction oxidises
the chromogen (PERID) to form a dye:

$$H_2O_2 \quad + \quad chromogen \longrightarrow Dye \quad + \quad H_2O$$
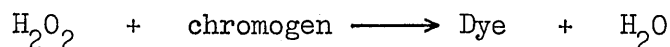
the intensity of the dye is proportional to the glucose con-
centration and was measured at a wavelength of 640 nm in a
Beckman Spectrophotometer (DB-GT, Beckman Instruments, INC.).
The standard curve for glucose was linear up to values of 19.43 mmol/l.

II.   Insulin assay:

Determinations of insulin concentrations in the collected
samples of each experiment were done in duplicate by a Sephadex-
adsorbed-antibody radioimmunoassay method (Phadebas Insulin Test-
Pharmacia Diagnostics) where the separation of bound from unbound
insulin is carried out by the specific antibodies coupled by
covalent linkages to a solid phase which is the activated sephadex
particles suitable for radioimmunoassay (Wide & Porath 1966); after
incubation the bound and free antigen are separated by centrifugation.

Each Phadebas Insulin Test Kit contains the following reagents:

(1)   Sephadex-Anti-Insulin Complex, antibodies raised in
guineapigs (Lyophilized).

(2)   Insulin standard 320 µU/ml after reconstitution
(Lyophilized).

(3)   Insulin^{125}I : 8 ng; about 3 µCi (at date of
manufacture (Lyophilized).

(4)   Buffer substance, 4.2 gm (dry powder).

(5)   Phadebas Decanting-Aid-solution: 5 ml diluted to
250 ml using normal saline solution.

To prepare the standard curve, the insulin standard solution
(320 µU/ml) is diluted with buffer solution to obtain suitable con-
centrations within the range 5-320 µU/ml; performed by serial double

dilutions using the standard stock solution to obtain the following standard concentrations: 320, 160, 80, 40, 20, 10 and 5 µU/ml. The buffer is used as "zero" insulin sample. Plastic tubes (Polythene, LP4 round bottom tube, L./072, Luckham Ltd.) were used for the following test procedure:

(1)   0.1 ml of standards 5-320 µU/ml and "zero" insulin was pipetted into tubes (Set I) each in duplicate.

(2)   0.1 ml of unknowns was pipetted into tubes (Set II) each in duplicate.

(3)   0.1 ml of the insulin ^{125}I solution was pipetted into each of the tubes of sets I and II.

(4)   1.0 ml Sephadex Anti-Insulin complex suspension was pipetted into each of the tubes of Sets I and II.

(5)   The tubes of both sets were stoppered and maintained overnight at room temperature on a rotary incubator which served to keep the particles in suspension.

(6)   Tubes of both sets were centrifuged at 2000 Rev./min. in a refrigerated centrifuge (Mistral 2L, MSE), for two minutes at 4°C, in order to remove droplets from the stoppers.

(7)   1.5 ml of phadebas decanting-aid solution (used to speed up and facilitate precipitation) were added to all tubes of both sets.

(8)   Tubes of both sets were centrifuged at 2000 Rev./min.
for five minutes and at 4°C. The supernatants were de-
canted to the last drop.

(9)   Radioactivity in tubes of both sets, as well as
two tubes each containing 0.1 ml ^{125}I solution (total
activity) was determined by using a Well-type gamma-
counter (Gamma Sample Counter, GTL 300-500, Wallac Decem
Series). The anode tension was 1.086 KV, the discriminator
level was 2.18 volts, and the channel width (counting
window) was 1.8 volts. The efficiency of the counter was
55%. The counting time of each sample was 240 seconds.

III.   Evaluation of oxygen uptake:

The oxygen carrying capacity of the perfusion media was
assessed after an oxygen dissociation curve was established for a
given amount of perfusion medium containing the oxygen carrier
FX-80 (perfluoro-2-Butyl tetrahydrofurans). The amount of oxygen
taken up, i.e. the oxygen content of the medium in terms of $ml(O_2)$/100 ml
(medium) was related to the partial pressure of the oxygen (PO_2)
equilibrated with it.

The perfusion medium was put in the oxygenator of the
perfusion setup kept at a bath temperature of 37.5°C and was
oxygenated by a humidified gas mixture (O_2:CO_2, 2%:5%, 93% N_2)
for twenty five minutes after which a 2 ml sample was analysed for
total oxygen content by the Van Slyke method. The same procedure

was repeated for different gas mixtures containing 3.5, 5, 6.5, 8, 21 and 40% oxygen while keeping the CO_2 percentage constant at 5%, the rest was N_2. Total oxygen content of each sample was determined and expressed in (ml) of oxygen per (100 ml) perfusion medium at s.t.p. An oxygen dissociation curve was constructed accordingly by plotting total oxygen content versus corresponding partial pressures of oxygen (Appendix 4).

Prior to the start of perfusion in each experiment, partial pressures of oxygen in the perfusion media were measured by the oxygen electrode and these readings were considered to represent the arterial oxygen tension; the venous oxygen tensions were measured throughout the whole perfusion process by passing the venous outflow through the oxygen electrode. The arterial and the venous oxygen tensions were expressed in mm Hg, at $37.5^{\circ}C$.

IV.   Volume flow of perfusates:

The volume of each sample was calculated as the product of the number of drops collected for that sample by the volume of one drop, which was estimated after repeated precise measurements of the volume of one hundred drops of perfusion media passed through the same dropper head and was confirmed by comparison of weights of equal volumes of the same medium.

V.   Calculations and statistical analyses:

1.   Glucose concentration:

Calculation of the glucose concentration of the
prepared perfusion media as well as the collected samples
was based on the comparison of the opticaldensity (E) of
the unknown samples with the optical density of the
standard (5.55 mmol/l), hence the glucose concentration
was calculated from the expression $\frac{E \text{ (sample)}}{E \text{ (standard)}}$ x 5.55

The results were expressed in (mmol/l). The reported
concentrations of glucose in each experiment were the
averages of duplicates of each sample.

Differences between glucose concentrations of
perfusing media and corresponding samples collected from
each experiment, represented the glucose uptake by the
perfused block of tissue. Uptake was presented as
millimoles glucose per gram tissue per hour (mmol/g/h).

2.   Insulin secretion rate:

The count rate (CR) of radioactivity for each of
the standards was expressed as a percentage of the mean maximum
count rate (MCR) of the "zero" insulin sample after the
subtraction of the background counts. The percentage
values obtained for the insulin standards were plotted
against the logarithm of insulin concentration on semi-
logarithmic graph paper, thus a standard curve was con-
structed. Similarly, the average count rate for each of

the unknowns was expressed as a percentage of (MCR) in
the same way as for the standards. The concentration
of insulin was read from the standard curve (in $\mu U/ml$)
for each of the unknown samples. The insulin secretion
rate of each sample collected was expressed as micro
units of insulin per gram of wet weight of pancreas per
five minutes (sample collection time) i.e. $\mu U/g/5$ min.,
which was calculated as follows: $\dfrac{C \times V}{W}$ per 5 min.

where C = insulin concentration ($\mu U/ml$); V = volume of
sample (ml), W = Wet weight of pancreas (g). Results of
each experiment were plotted in a histogram of insulin
secretion rate versus time. The results of each set of
experiments were analysed statistically by grouping
replicate samples and calculating their means and standard
errors of the means (s.e) and were plotted in a histogram
of the mean value of insulin secretion rate of each sample
$\pm$ s.e. versus time in minutes.

3. The student-t-test:

The final analysis of the research, intended to compare
the means of data obtained from the different sets of
experiments performed with different conditions and/or
different experimental parameters, was the student-t-test
for matched paired samples (two tailed test), which
compared two mean values of insulin secretion rates of
comparable standing and showed whether they were significantly
different or not, taking into consideration their variances.

The critical significances point in these analyses was taken at probability $p \leq 0.05$, at the corresponding degrees of freedom (df) of the number of replicates of each value concerned.

RESULTS

The results of the six sets of experiments are presented here in the order in which the different categories of experimental animals were discussed earlier. A typical insulin assay standard curve and a general comment on the measurement of oxygen uptake are included prior to the experimental results.

I.   Insulin assay standard curve:

Standard curves of insulin concentration versus percentage activity were plotted on semilogarithmic graph paper and all conformed to the typical pattern shown in Fig. (4). Standards of 320 μU insulin/ml showed 25 $\pm$ 1.5 (Mean $\pm$ s.e.) % of total radioactivity bound and the 5 μU insulin/ml showed 90.5 $\pm$ 1.0 (Mean $\pm$ s.e.) % of total radioactivity bound. None of the experimental samples fell outside the above range of standards.

The standard insulin used was porcine insulin Fig. (4) and the insulin antibody of the sephadex-anti insulin complex was raised in guineapigs. A rat standard insulin sample 20.7 $\pm$ 3 I.U./mg (generously supplied by Mr. R. L. Cooper, Diagnostics Division, Pharmacia (G.B.) Ltd.) was tested for cross reactivity with the Sephadex-anti insulin complex of the insulin assay kit. The examination showed a higher binding capacity by the rat insulin than that of the porcine insulin for values more than 20 μU/ml, and lower for values less than 20 μU/ml Fig. (4). Results of insulin concentrations in all the experiments were expressed in micro

% Activity bound

μU Insulin/ml
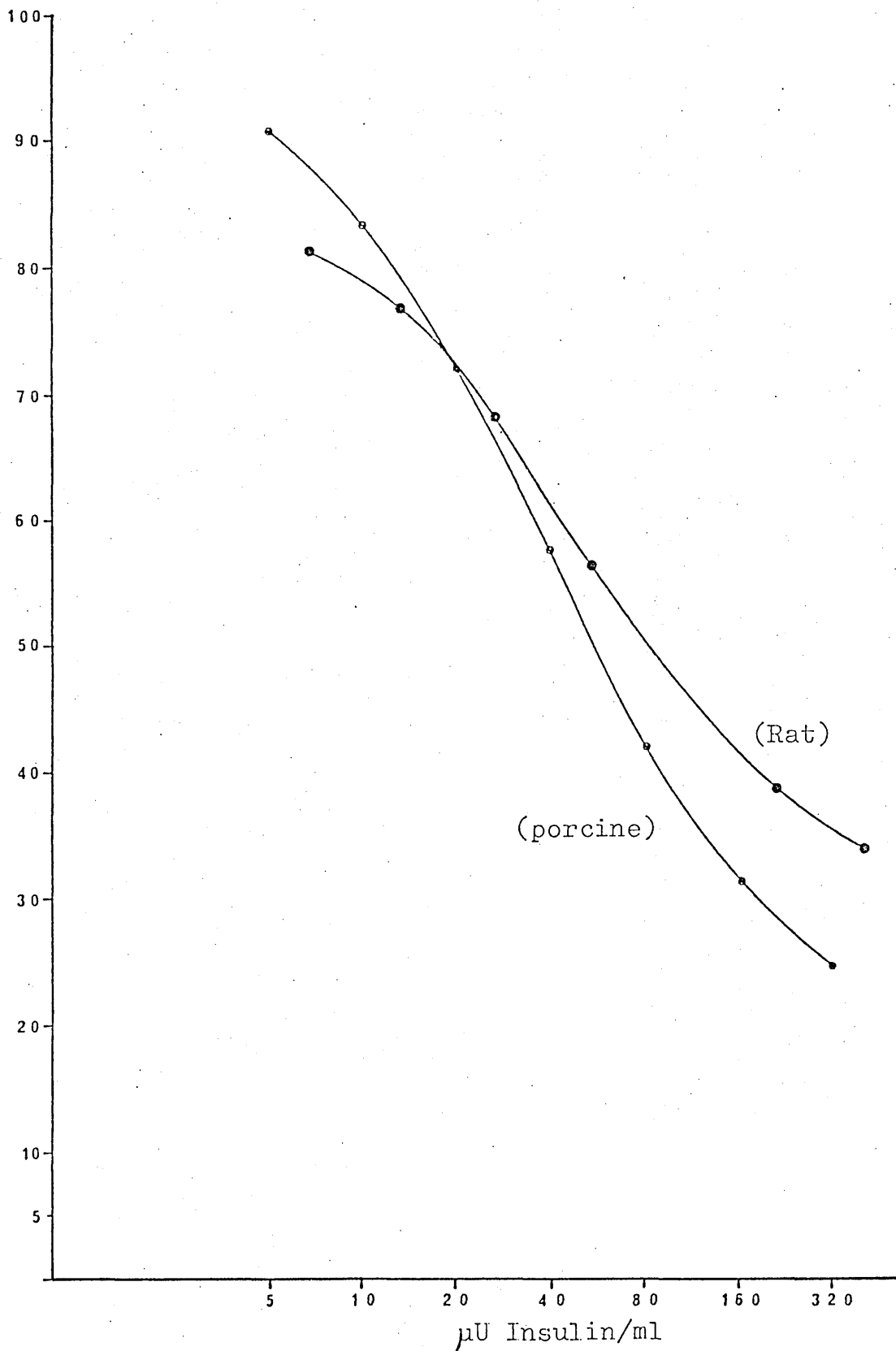
Figure 4. Standard curves for rat and porcine insulin as obtained from radioimmunoassay.

international units of insulin per millilitre perfusate ($\mu U/ml$)
as equivalents of porcine insulin. This reflects relative change
of insulin concentration values and serves the purpose of the
investigation adequately.

II.   Oxygen uptake:

In all the pancreas perfusion experiments performed, the
"arterio-venous" (i.e. input-output) oxygen tension differences
ranged between 240 and 260 mm Hg. The perfusate "arterial" (input)
oxygen tension, after thirty minutes of equilibration with O_2:CO_2
(95%:5%), ranged between 345 and 365 mm Hg (Mean $\pm$ s.e. =
360 mm Hg $\pm$ 2.6); on the other hand the "venous" (output) oxygen
tension was 106 mm Hg $\pm$ 2.3. Precise values of oxygen uptake by
the perfused block of tissue were not calculated because this arterio-
venous oxygen tension difference did not demonstrate a net oxygen
delivery with reference to the oxygen dissociation curve constructed
for the perfusion media with (FX-80) as the oxygen carrier
(Appendix 4). Nevertheless, oxygen carried physically in a dissolved
form in the perfusion medium seems to have been enough to sustain the
perfused block of tissue in a normal physiological state for a total
perfusion period of two hours and exhibiting uptake of glucose,
insulin release in response to glucose stimulation and peristaltic
movements of the duodenum.

III.   Insulin secretion in the main control group:

Results of the main control groups, i.e. from non cold-
exposed animals, of pancreas perfusion experiments are summarized

Table 1: Data obtained from outflow of perfused
pancreases of the main control set of rats

Sample	Volume ml	Glucose concentration mmol/l	Insulin concentration µU/ml	Insulin secretion rate µU/g/5 min.
1	2.15 ± 0.03	3.71 ± 0.17	38.75 ± 5.09	94.51 ± 11.68
2	2.14 ± 0.04	3.67 ± 0.17	38.58 ± 6.07	94.34 ± 14.16
3	2.14 ± 0.04	3.83 ± 0.28	39.78 ± 6.51	97.43 ± 15.57
4	2.145± 0.04	4.07 ± 0.27	39.63 ± 5.48	97.15 ± 12.89
5	2.15 ± 0.03	4.34 ± 0.22	41.33 ± 5.25	101.38 ± 11.92
6	2.12 ± 0.04	4.85 ± 0.33	47.58 ± 7.21	115.50 ± 4.64
7	2.14 ± 0.03	9.11 ± 0.69	59.20 ± 7.16	144.61 ± 15.51
8	2.15 ± 0.03	12.55 ± 0.69	73.17 ± 4.98	179.44 ± 8.57
9	2.15 ± 0.04	12.29 ± 0.64	60.50 ± 4.47	148.22 ± 7.39
10	2.12 ± 0.03	13.73 ± 0.58	57.33 ± 7.05	139.09 ± 10.53
11	2.13 ± 0.02	13.96 ± 0.53	63.33 ± 6.59	153.82 ± 9.09
12	2.15 ± 0.03	13.85 ± 0.40	67.88 ± 4.08	166.43 ± 3.60
13	2.15 ± 0.03	13.79 ± 0.44	62.75 ± 4.67	154.36 ± 9.74
14	1.95 ± 0.04	13.31 ± 0.69	65.13 ± 8.95	144.80 ± 12.45
15	2.15 ± 0.03	13.36 ± 0.65	54.42 ± 6.99	133.99 ± 16.09
16	2.15 ± 0.03	13.11 ± 0.62	55.90 ± 5.99	137.21 ± 12.92
17	2.14 ± 0.04	13.95 ± 0.62	61.92 ± 8.10	151.22 ± 16.92
18	2.13 ± 0.03	13.20 ± 0.60	55.87 ± 6.03	136.25 ± 11.28
19	2.15 ± 0.03	13.31 ± 0.68	58.90 ± 7.07	144.73 ± 14.10
20	2.15 ± 0.03	13.38 ± 0.62	58.59 ± 5.83	143.96 ± 12.92

i)   In all cases, n = 6
ii)   Mean body weight of animals = 246.33 ± 8.34 g
iii)   Mean wet weight of pancreas = 0.875 ± 0.40 g
iv)   All values are expressed as the mean and the standard error of
the mean.
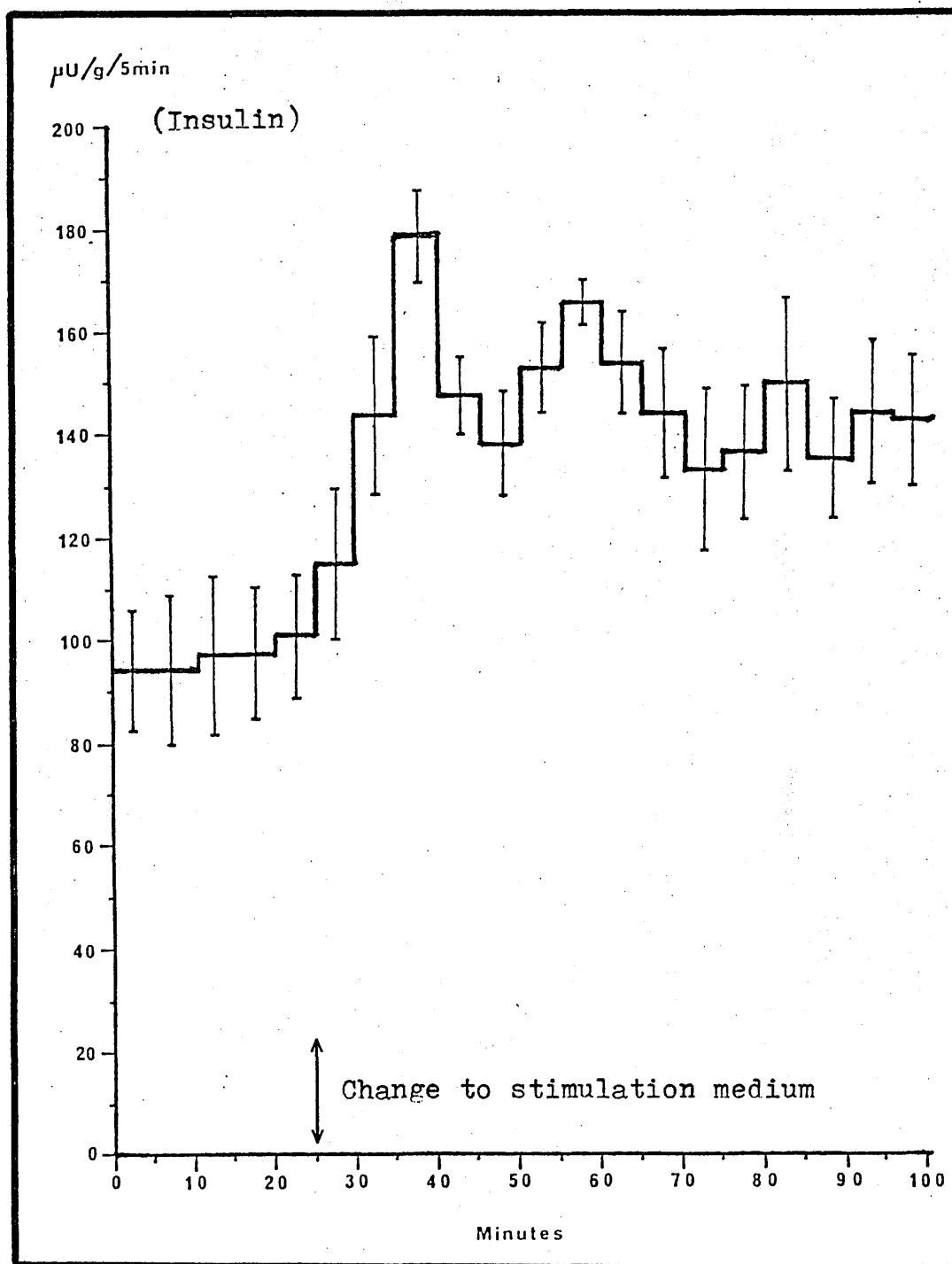
Figure 5. Histogram of insulin secretion rates vs. time, in the main control group, showing the basal rate and the biphasic insulin secretion rates in response to glucose stimulation. Values are the means of six samples and the vertical bars represent the standard errors of the means in each case.

in Table (1) and Fig. (5). Six rats with body weight 246.3 ± 8.3
(s.e.m.) g were used to prepare the perfusion assembly with a mean
pancreatic wet weight of 0.875 ± 0.040 g. The average perfusion
flow rate was 2.13 ± 0.01 ml/5 min. and the perfusion pressure
ranged between 80 and 94 mm Hg. Glucose concentration in the
basal perfusion medium was 3.92 ± 0.11 mmol/l (70.63 ± 1.98 mg%);
and in the stimulation (i.e. high glucose concentration) perfusion
medium, glucose concentration was 13.54 ± 0.13 mmol/l
(243.97 ± 2.34 mg%).

The basal insulin secretion rate (at zero to twenty five
minutes perfusion time) ranged between 94.34 ± 14.16 μU/g/5 min.
and 101.38 ± 11.92 μU/g/5 min., i.e. in the first five samples
collected during the 25 min. of basal medium perfusion. Following
a change from basal to stimulation medium, the insulin secretion
rate increased and perfusion with the stimulation medium was
maintained for a further 75 minutes, after which the experiment
was concluded. The expected biphasic secretion pattern of insulin
(Basake et al., 1971; Bennett, Curry and Curry, 1973; Cerasi and Luft,
1967; Curry, 1971; Fussganger, Hinz, Goberna, Jaros, Karsten,
Pfeiffer & Raptis, 1969; Gerhards and Ruel, 1974; Grodsky et al.,
1963, 1970; Katada and Ui, 1977; Kidson and Lazarus, 1973;
Lenzen and Hasselblatt, 1974; Lerner, 1977; Loubatières, Alric,
Mariani and Chapal, 1972; Loubatières and Loubatières-Mariani, 1974;
Pfeiffer, Fussganger, Hinz and Raptis, 1970) is evident after a
glucose load of 8.33 mmol/l or more was applied, Fig. (5). A peak
pulse of insulin release 179.44 ± 8.57 μU/g/5 min. (mean ± s.e.) was

achieved within five minutes of the stimulation medium reaching the pancreas. This first phase of insulin release was followed by a drop in the rate of release to 139.09 ± 10.53 µU/g/5 min., after which the second phase commenced and remained within the range 133.99 ± 16.09 to 166.43 ± 3.60 µU/g/5 min. during the period of observation. The insulin secretion rates of both phases are significantly greater than that seen during perfusion with the basal medium (p < 0.001). In response to glucose stimulation, insulin released in the first phase of secretion, which lasted for approximately five minutes, is about 10% of that released during the observed fifty minutes of the second phase.

Glucose uptake by the block of perfused tissue (which included the major part of the pancreas, a segment of the duodenum and some fat in close association with the pancreatic tissue) was observed to differ, depending on whether the basal medium or the stimulation medium was perfusing the block of tissue. The glucose uptake was estimated from the following values:

a - Average glucose concentration in basal medium .... 4.506 mmol/l.

b - Average glucose concentration in stimulation medium .... 16.750 mmol/l.

c - Average glucose concentration in the first five samples .... 3.924 mmol/l.

d - Average glucose concentration in the last ten samples .... 13.540 mmol/l.

e   -   Average volume of samples

.... 2.132 ml/5 min.

f   -   Average wet weight of perfused tissue

.... 2 g.

Glucose uptake from basal medium = 7.445×10^{-3} mmol/g/hour;

and glucose uptake from the stimulation medium = 4.106×10^{-2}

mmol/g/hour.

IV.   Insulin secretion in cold-acclimated animals:

The rats, undergoing cold-acclimation at $4^\circ C$, tolerated the

low temperature successfully, mortality rate being 4%. Results of

the pancreas perfusion experiments from the cold-acclimated rats

are summarized in Table (2) and Fig. (6).

Six rats of average body weight of 439.5 ± 7.9 g were

used to prepare the isolated perfused pancreases which had a mean

wet weight of 1.119 ± 0.0941 (s.e.m.) g. Average perfusion flow

rate was 1.14 ml/5 min and the perfusion pressure ranged between

90 and 95 mm Hg. Glucose concentration in the basal perfusion

medium was 4.25 mmol/l, and in the stimulation perfusion medium

glucose concentration was 15.91 mmol/l. The basal rate of insulin

release ranged between 19.50 ± 6.61 µU/g/5 min. and 25.70 ± 5.59

µU/g/5 min. The biphasic pattern of insulin release in response

to glucose stimulation is evident: the first phase of release peaked

at 38.60 ± 5.02 µU/g/5 min. and occurred within 15 minutes of the

shift into the high glucose load of the stimulation medium; the second

phase of insulin release appeared ten to fifteen minutes after the

first phase and ranged between 27.10 ± 3.51 µU/g/5 min. and

Table 2: Data obtained from outflow of perfused
pancreases of the cold-acclimated set of rats

Sample	Volume ml	Glucose concentration mmol/l	Insulin concentration µU/ml	Insulin secretion rate µU/g/5 min.
1	1.24 ± 0.13	3.32 ± 0.15	24.80 ± 8.83	23.30 ± 5.88
2	1.20 ± 0.13	3.42 ± 0.15	21.90 ± 9.46	19.50 ± 6.61
3	1.18 ± 0.13	3.55 ± 0.11	24.48 ± 9.41	20.90 ± 5.39
4	1.20 ± 0.13	3.65 ± 0.13	26.88 ± 10.01	23.80 ± 5.67
5	1.26 ± 0.14	3.85 ± 0.19	28.37 ± 10.20	25.70 ± 5.59
6	1.18 ± 0.13	5.01 ± 0.53	31.92 ± 9.85	28.40 ± 4.94
7	1.12 ± 0.13	7.34 ± 0.83	35.13 ± 9.65	29.90 ± 4.94
8	1.13 ± 0.15	12.75 ± 0.50	44.66 ± 9.53	38.60 ± 5.02
9	1.16 ± 0.16	12.91 ± 0.37	35.95 ± 10.43	30.70 ± 4.41
10	1.12 ± 0.14	13.22 ± 0.34	33.08 ± 10.22	27.90 ± 4.33
11	1.10 ± 0.14	13.46 ± 0.26	31.10 ± 10.08	25.50 ± 4.49
12	1.09 ± 0.15	13.63 ± 0.34	34.33 ± 10.84	27.10 ± 3.51
13	1.11 ± 0.14	13.51 ± 0.34	35.13 ± 10.16	29.60 ± 4.41
14	1.10 ± 0.15	13.05 ± 0.54	33.63 ± 10.37	27.10 ± 3.67
15	1.07 ± 0.15	13.84 ± 0.33	35.37 ± 10.23	27.90 ± 3.76
16	1.08 ± 0.14	13.85 ± 0.26	37.12 ± 10.00	30.40 ± 4.86
17	1.08 ± 0.13	13.14 ± 0.34	37.83 ± 9.59	31.30 ± 5.31
18	1.09 ± 0.13	13.11 ± 0.35	32.33 ± 9.24	26.90 ± 4.78
19	1.11 ± 0.13	13.34 ± 0.29	34.61 ± 9.52	29.36 ± 3.95
20	1.09 ± 0.13	13.22 ± 0.36	33.80 ± 8.95	28.15 ± 3.75

i) In all cases, $n = 6$
ii) Mean body weight of animals $= 439.5 \pm 7.92$ g
iii) Mean wet weight of pancreas $= 1.1185 \pm 0.094$ g
iv) All values are expressed as the mean and the standard error of
the mean.
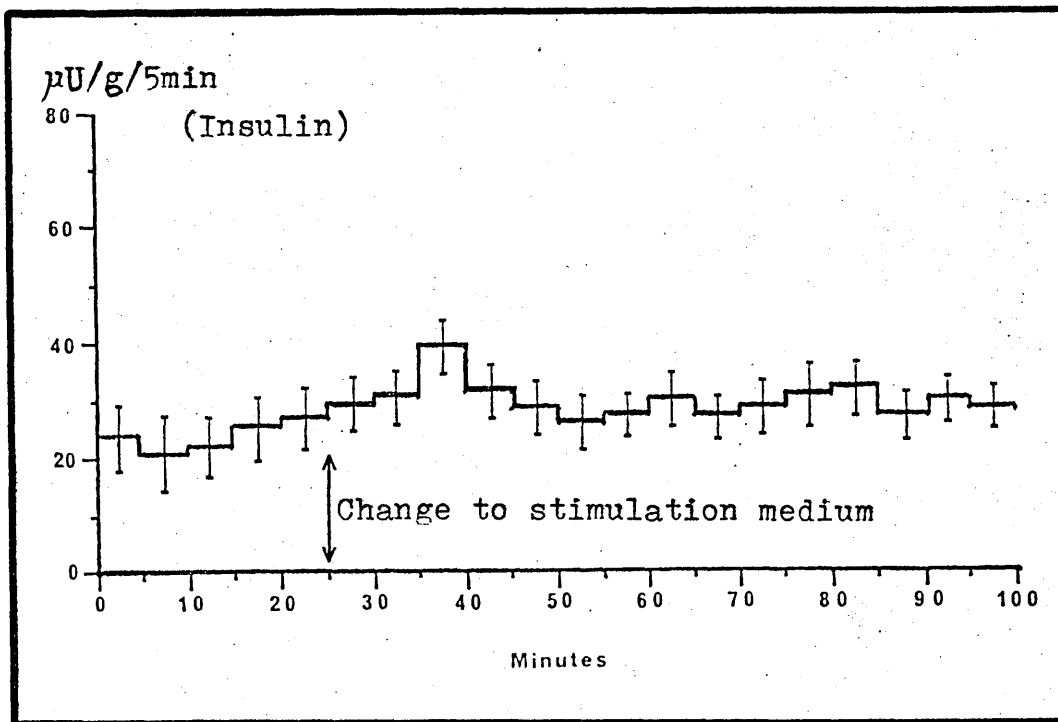
Figure 6. Histogram of insulin secretion rates vs. time, in the
cold-acclimated group, showing the basal rate and the
effect of glucose stimulation on insulin release.
Values are the means of six samples, and the vertical
bars represent the standard errors of the means, in
each case.

31.30 ± 5.31 µU/g/5 min. The differences between the insulin secretion rate at the peak of the first phase and those of the basal rate of release were highly significant ($p < 0.001$) and the differences between the rate of insulin release of the peak of the second phase and all samples of the basal rate were significant ($p < 0.05$). Insulin released in the first phase after glucose stimulation was about 12% of that released during the second phase for 45 minutes.

Glucose uptake by the block of perfused tissue was 4.717×10^{-3} mmol/g/hour from the basal medium and 1.713×10^{-2} mmol/g/hour from the stimulation medium.

V.   Noradrenaline dose-response effect on insulin secretion

The purpose of this set of experiments is to study the effect of locally applied noradrenaline on insulin release from the pancreatic islets and to evaluate quantitavely the amount of noradrenaline needed for maximal effect.

Results of the five sets of experiments, each set comprising six preparations and at a different noradrenaline concentration in the perfusion media, are summarized in Table (3) and Fig. (7).

With the noradrenaline concentration at 5 ng/100 ml of perfusate, the insulin release in response to glucose load followed the general biphasic pattern Fig. (7-a). The basal insulin secretion rate during the perfusion of the basal medium ranged between 85.2 ± 0.6 µU/g/5 min. and 97.7 ± 7.5 µU/g/5 min.

Table 3: Data obtained from outflow of pancreases perfused with different
noradrenaline (NA) concentrations. Pancreases were isolated from normal control rats

Insulin Secretion Rates µU/g/5 min.

Sample	5ng(NA)/100 ml	20ng(NA)/100 ml	40ng(NA)/100 ml	80ng(NA)/100 ml	100ng(NA)/100 ml
1	90.5 ± 2.2	76.4 ± 2.0	59.8 ± 4.1	52.4 ± 1.4	52.8 ± 1.4
2	85.2 ± 0.6	62.8 ± 1.7	59.7 ± 4.7	51.7 ± 1.4	52.4 ± 1.7
3	94.5 ± 8.2	70.7 ± 0.7	63.5 ± 4.2	51.4 ± 0.8	53.6 ± 1.7
4	92.8 ± 5.2	69.0 ± 2.0	58.2 ± 2.1	51.5 ± 1.0	52.7 ± 1.3
5	89.7 ± 2.3	72.2 ± 2.1	58.2 ± 4.8	52.5 ± 1.2	53.4 ± 1.2
6	91.0 ± 5.3	79.2 ± 5.4	60.2 ± 1.2	55.7 ± 2.1	53.2 ± 2.6
7	97.7 ± 7.5	92.8 ± 2.5	73.4 ± .06	56.6 ± 2.0	54.1 ± 2.4
8	129.6 ± 4.7	100.6 ± 2.4	86.2 ± 2.0	58.9 ± 2.8	55.0 ± 2.3
9	87.9 ± 1.0	81.6 ± 1.5	69.4 ± 2.3	59.0 ± 1.9	55.1 ± 1.9
10	99.3 ± 10.1	81.8 ± 3.5	70.6 ± 1.1	63.0 ± 2.1	55.5 ± 1.6
11	103.2 ± 10.4	87.7 ± 3.1	72.5 ± 1.5	69.9 ± 3.0	56.2 ± 1.6
12	105.8 ± 9.0	91.2 ± 2.7	67.3 ± 1.7	74.5 ± 3.7	56.7 ± 2.4
13	121.7 ± 8.0	93.7 ± 2.9	75.5 ± 1.6	73.9 ± 1.1	59.3 ± 4.1
14	115.7 ± 7.4	95.3 ± 1.8	75.6 ± 2.5	72.2 ± 1.5	61.6 ± 4.5
15	114.9 ± 1.4	94.0 ± 1.3	77.4 ± 2.0	64.2 ± 1.5	62.3 ± 6.1
16	126.4 ± 8.4	95.1 ± 2.2	76.2 ± 2.5	73.3 ± 0.9	63.2 ± 5.3
17	129.9 ± 5.5	95.4 ± 0.8	77.2 ± 2.6	72.2 ± 1.0	62.7 ± 5.8
18	128.6 ± 3.5	94.4 ± 0.9	77.1 ± 2.4	74.2 ± 0.6	63.1 ± 5.7
19	143.2 ± 10.7	92.2 ± 0.6	75.8 ± 1.4	72.7 ± 1.2	63.4 ± 5.1
20	145.6 ± 5.5	92.7 ± 0.5	75.0 ± 1.8	72.7 ± 0.5	63.5 ± 6.3

i) In all cases, n = 6
ii) All values are expressed as the mean and standard error of the mean.
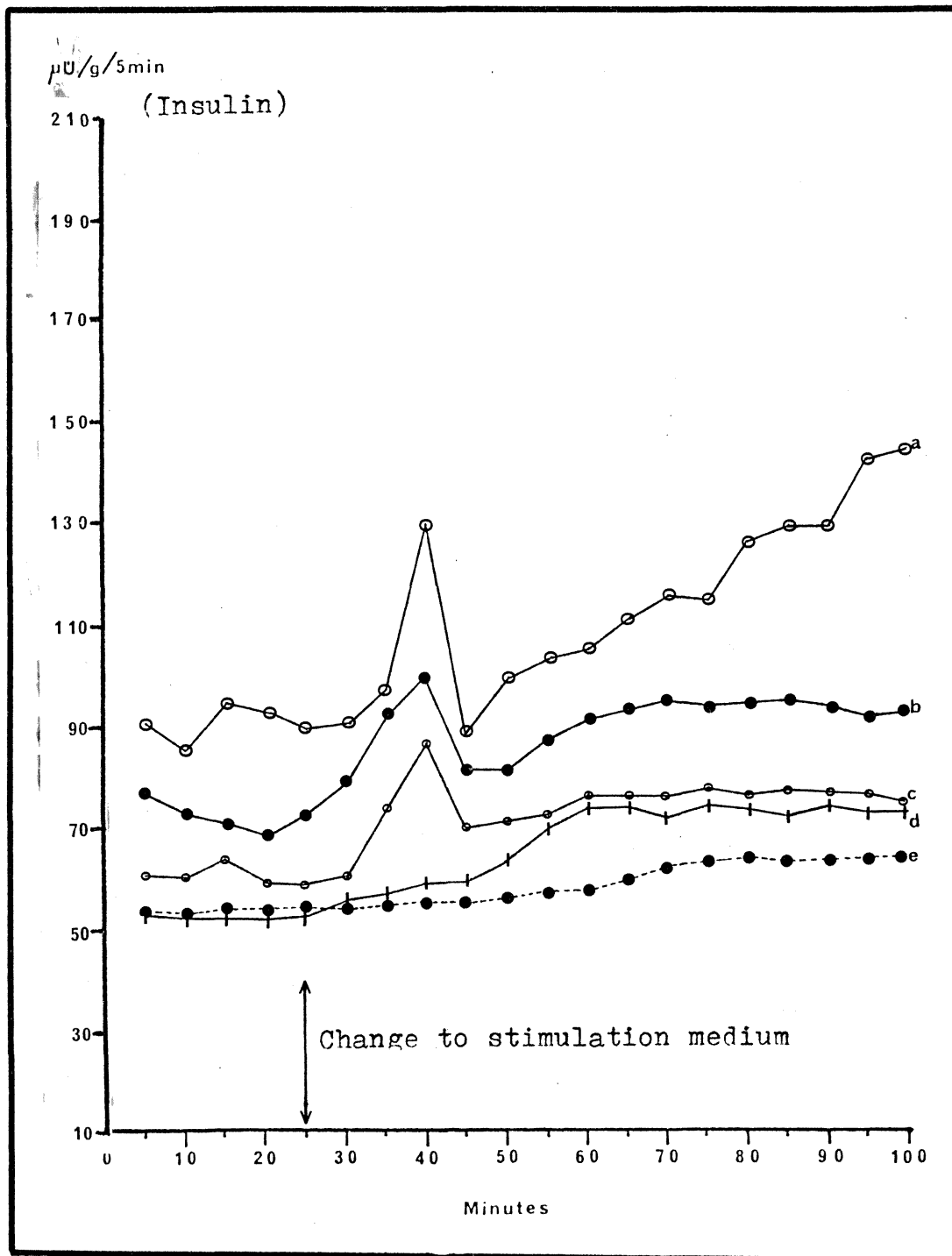
Figure 7.   Noradrenaline dose-effect on insulin secretion rates

basal and in response to glucose stimulation.

Noradrenaline concentrations included in both

perfusion media were:

(a) 5, (b) 20, (c) 40, (d) 80 and (e) 100 ng/100 ml.

Each point represents the mean of six samples.

The first phase of insulin release, in response to glucose stimulation, peaked at 129.6 ± 4.7 $\mu U/g/5$ min., which sub-subsided after five minutes. The second phase of insulin release ranged between 103.2 ± 10.4 $\mu U/g/5$ min. and 145.6 ± 5.5 $\mu U/g/5$ min. The overall result, by point to point comparison, was a slight supression of insulin release in comparison with the normal control set. Nevertheless, the first six samples which denoted the basal insulin release were found to be not significantly different $(p \geq 0.5)$ from the normal control set; the first phase of insulin release on the next four samples, were significantly lower than the control $(p < 0.02)$; the second phase of insulin release on the last eight samples were not significantly different $(p \geq 0.5)$ from the control.

Increasing the noradrenaline concentration to 20 ng/100 ml perfusate Fig. (7-b), and to 40 ng/100 ml perfusate Fig. (7-c), resulted in a further suppression of insulin release, but the biphasic pattern of insulin release was still persistant. The basal insulin release, during the perfusion with 20 ng/100 ml noradrenaline, was not significantly different from the control; whereas with 40 ng/100 ml noradrenaline infusion the basal insulin release was significantly lower than the control $(p < 0.04)$. Insulin secretion rates in response to glucose stimulation in the presence of either 20 ng or 40 ng noradrenaline per 100 ml perfusion media, were all significantly lower than the control (both phases of release) $(P < 0.01)$.

When the noradrenaline concentration was increased to

80 ng/100 ml perfusate, Fig. (7-d), and still higher to 100 ng/100 ml

perfusate, Fig. (7-e), the pattern of insulin release was completely

changed. The biphasic pattern was abolished and there was no

evidence of a sharp first phase of release. However during the

80 ng noradrenaline per 100 ml perfusion, the glucose loads of

16.14 and 16.32 mmol/l had an overall stimulating effect which

resulted in a slight increase in the rate of insulin release ranging

between 63.0 ± 2.1 and 72.7 ± 0.5 μU/g/5 min. These insulin

secretion rates were significantly higher than the basal insulin

secretion rates of the same set (p < 0.02). During the 100 ng

noradrenaline per 100 ml perfusion, the stimulated insulin secretion

rates ranged between 55.5 ± 1.6 and 63.5 ± 6.3 μU/g/5 min., which in

comparison with the basal secretion rates of the same set that

ranged between 51.4 ± 0.8 and 53.4 ± 1.2 μU/g/5 min., were slightly

higher (p < 0.05). All values of insulin secretion rates of both

sets of experiments at 80 ng and 100 ng noradrenaline per 100 ml

perfusate, were significantly lower than corresponding values

(basal, first and second phases) of insulin secretion rates of the

main control group (P < 0.001).

VI.   Insulin secretion after cold-acclimation and in the presence
       of noradrenaline and phentolamine in the perfusates

Following the noradrenaline dose-response control experiments,

the next set was performed with noradrenaline (100 ng/100 ml) in

both the basal and the stimulation media, together with phentolamine

(50 µg/100 ml) the alpha-adrenergic antagonist, which was
expected to abolish the inhibitory effect of noradrenaline on
insulin release from the pancreatic islets.

Results of this set of experiments are summarized in
Table (4) and Fig. (8). Six rats with an average body weight of
309 ± 6.2 g were used to prepare the pancreas perfusions. The
average wet weight of the perfused pancreases was 0.871 ±
0.044 g. The average perfusion flow rate was 1.90 ± 0.02 ml/5 min.
The perfusion pressure ranged between 92 and 108 mm Hg. Glucose
concentration in the basal medium was 4.15 mmol/l and in the
stimulation medium was 15.14 mmol/l. The basal rate of insulin
secretion ranged between 28.35 ± 7.39 µU/g/5 min. and 38.17 ±
6.53 µU/g/5 min. Stimulation with the glucose load resulted in
a first phase of insulin release which peaked at 51.38 ± 7.96
µU/g/5 min. and then subsided to lead into a second phase of insulin
release ranging between 38.72 ± 8.82 µU/g/5 min. and 47.82 ± 7.51
µU/g/5 min. Thus, the general biphasic secretion pattern of
insulin once again became evident. The differences between the
mean values of the peak of the first phase and those of the basal
secretion rate are highly significant (p < 0.001), and the differences
between the mean values of the second phase of insulin release and
those of the basal range are significant (p < 0.05). Insulin released
in the first phase after glucose stimulation was about 13.4% of that
released during the second phase of release for forty five minutes.
Glucose uptake by the block of perfused tissue was calculated to be
2.46×10^{-3} mmol/g/hour from the basal perfusion medium and 1.255 x
10^{-2} mmol/g/hour from the stimulation medium.

Table 4: Data obtained from outflow of pancreases
in the presence of both noradrenaline and
phentolamine in both perfusion media. Pancreases were isolated
from cold-acclimated rats

Sample	Volume ml	Glucose concentration mmol/1	Insulin concentration μU/ml	Insulin secretion rate μU/g/5 min.
1	1.97 ± 0.06	3.91 ± 0.09	13.12 ± 3.14	30.23 ± 7.37
2	1.95 ± 0.05	3.93 ± 0.07	12.57 ± 3.22	28.35 ± 7.39
3	1.87 ± 0.07	3.96 ± 0.08	13.35 ± 3.17	29.05 ± 7.10
4	1.94 ± 0.09	3.92 ± 0.06	15.02 ± 2.74	33.50 ± 6.08
5	1.98 ± 0.08	3.95 ± 0.05	15.77 ± 2.56	35.82 ± 5.96
6	1.92 ± 0.09	6.61 ± 0.18	17.28 ± 2.95	38.17 ± 6.53
7	1.89 ± 0.06	11.25 ± 0.26	21.37 ± 3.61	46.43 ± 7.68
8	1.90 ± 0.06	13.76 ± 0.25	23.60 ± 3.81	51.38 ± 7.96
9	1.87 ± 0.05	14.08 ± 0.25	19.43 ± 2.96	41.77 ± 6.08
10	1.93 ± 0.07	14.08 ± 0.24	16.38 ± 3.06	35.97 ± 6.41
11	1.89 ± 0.04	14.06 ± 0.24	16.73 ± 3.25	36.10 ± 6.65
12	1.93 ± 0.04	14.06 ± 0.25	17.45 ± 3.90	38.22 ± 8.16
13	1.88 ± 0.04	14.05 ± 0.23	21.75 ± 3.97	46.43 ± 7.80
14	1.88 ± 0.04	13.98 ± 0.26	22.18 ± 3.64	47.82 ± 7.51
15	1.91 ± 0.06	14.03 ± 0.25	21.68 ± 3.88	37.65 ± 8.21
16	1.88 ± 0.04	14.03 ± 0.27	20.97 ± 3.74	46.00 ± 8.08
17	1.87 ± 0.04	13.98 ± 0.27	18.60 ± 4.63	39.05 ± 8.98
18	1.87 ± 0.06	14.02 ± 0.25	19.17 ± 4.27	40.98 ± 8.74
19	1.83 ± 0.05	14.06 ± 0.26	18.75 ± 4.76	39.55 ± 9.76
20	1.85 ± 0.04	14.06 ± 0.25	18.20 ± 4.24	38.72 ± 8.82

   i)   In all cases, n = 6
  ii)   Mean body weight of animals = 309 ± 6.21 g
 iii)   Mean wet weight of pancreas = 0.871 ± 0.044 g
  iv)   All values are expressed as the mean and the standard error of
                                                    the mean.
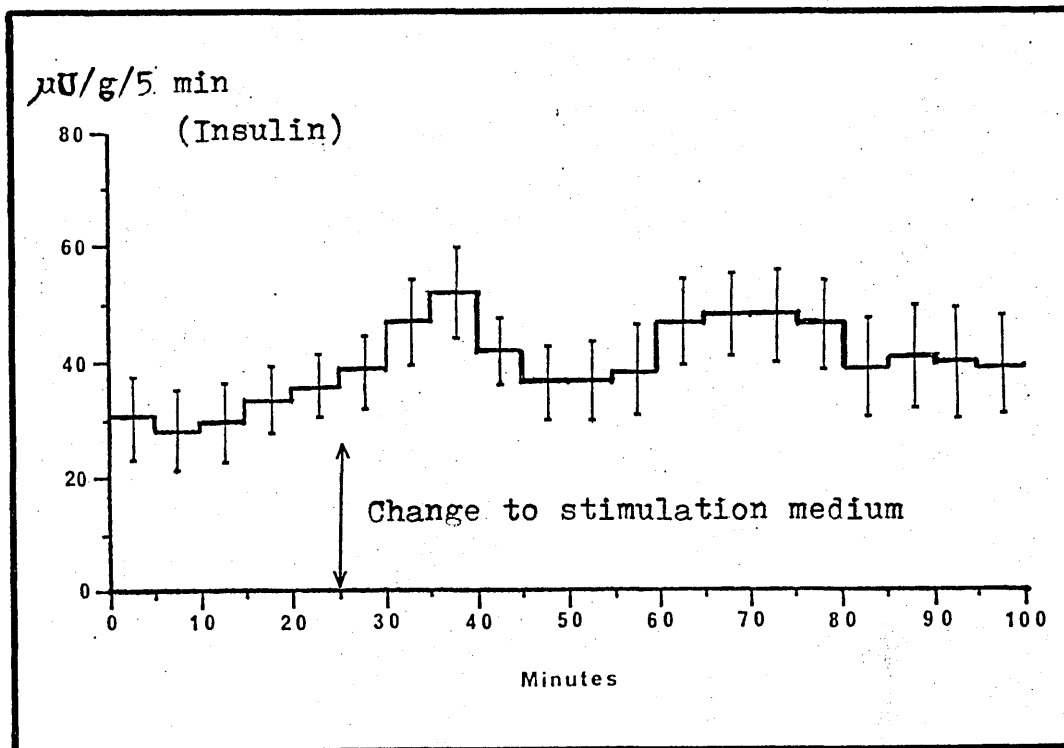
Figure 8.   Effect of noradrenaline (100 ng/100 ml) and phento-
lamine (50 µg/100 ml) on basal and glucose-stimulated
insulin secretion rates in the cold-acclimated rat
pancreas. Values are the means of six samples, and
the vertical bars represent the standard errors of
the means in each case.

VII.   Insulin secretion after phentolamine treatment during
        the last week of cold-acclimation of rats

        Results are summarized in Table (5) and Fig. (9). The
pancreases of six rats with an average body weight of 301.67
± 5.33 g, were isolated and perfused for this set of experiments.
The average wet weight of the pancreases was 0.671 ± 0.041 g.
The average perfusion rate was 1.51 ± 0.03 ml/5 min. and the
perfusion pressure ranged between 82 and 95 mm Hg. Glucose con-
centrations in the basal medium and in the stimulation medium
were 4.485 mmol/l and 16.94 mmol/l respectively.

        The basal rate of insulin release ranged between 34.07 ±
3.13 µU/g/5 min. and 43.43 ± 8.30 µU/g/5 min. In response to
glucose stimulation, the insulin release followed the general
biphasic pattern: the first phase of release peaked at 72.18 ±
10.04 µU/g/5 min. The second phase ranged between 59.52 ± 9.79
and 84.62 ± 7.50 µU/g/5 min. The differences between the values
of both phases of insulin release and the range of the basal
rate of insulin release are all highly significant ($p < 0.001$).
Insulin released in the first phase was about 9.3% of that
released during the second phase for fifty minutes.

        Glucose uptake by the block of perfused tissue was cal-
culated to be 4.626×10^{-3} mmol/g/hour from the basal perfusion
medium and 2.475×10^{-2} mmol/g/hour from the stimulation medium.

Table 5: Data obtained from outflow of perfused pancreases
of cold-acclimated rats treated with phentolamine during
the last week of cold-acclimation

Sample	Volume ml	Glucose concentration mmol/l	Insulin concentration μU/ml	Insulin secretion rate μU/g/5 min.
1	1.63 ± 0.19	3.82 ± 0.11	16.13 ± 3.09	34.07 ± 3.13
2	1.61 ± 0.18	3.80 ± 0.11	18.23 ± 5.25	37.17 ± 7.05
3	1.55 ± 0.20	3.81 ± 0.11	20.70 ± 6.46	41.08 ± 8.74
4	1.55 ± 0.19	3.79 ± 0.10	19.83 ± 5.74	39.20 ± 7.75
5	1.55 ± 0.20	3.85 ± 0.11	21.77 ± 6.10	43.43 ± 8.30
6	1.61 ± 0.19	7.08 ± 0.58	24.77 ± 6.04	51.52 ± 8.97
7	1.55 ± 0.18	12.09 ± 0.53	28.13 ± 7.54	57.45 ± 11.16
8	1.55 ± 0.17	13.21 ± 0.28	35.43 ± 7.91	72.18 ± 10.04
9	1.54 ± 0.17	13.96 ± 0.22	25.75 ± 5.20	53.62 ± 6.59
10	1.51 ± 0.18	13.98 ± 0.20	30.35 ± 7.13	59.52 ± 9.79
11	1.50 ± 0.19	13.99 ± 0.16	31.97 ± 7.30	61.15 ± 9.75
12	1.51 ± 0.18	14.32 ± 0.31	35.83 ± 7.27	71.37 ± 9.03
13	1.42 ± 0.12	14.12 ± 0.17	34.62 ± 6.01	68.23 ± 7.38
14	1.43 ± 0.11	14.20 ± 0.20	39.57 ± 6.22	79.25 ± 9.11
15	1.43 ± 0.12	14.38 ± 0.23	39.68 ± 6.69	79.03 ± 8.74
16	1.43 ± 0.11	14.36 ± 0.21	42.02 ± 7.07	94.23 ± 7.79
17	1.46 ± 0.11	14.14 ± 0.15	40.20 ± 5.07	84.62 ± 7.50
18	1.48 ± 0.11	14.42 ± 0.24	39.55 ± 6.20	82.48 ± 7.39
19	1.45 ± 0.11	14.23 ± 0.17	39.12 ± 5.04	82.15 ± 7.20
20	1.47 ± 0.11	14.15 ± 0.14	39.40 ± 5.21	83.37 ± 7.26

i) In all cases, n = 6
ii) Mean body weight of animals = 301.67 ± 5.33 g
iii) Mean wet weight of pancreas = 0.671 ± 0.041 g
iv) All values are expressed as the mean and the standard error of
the mean.
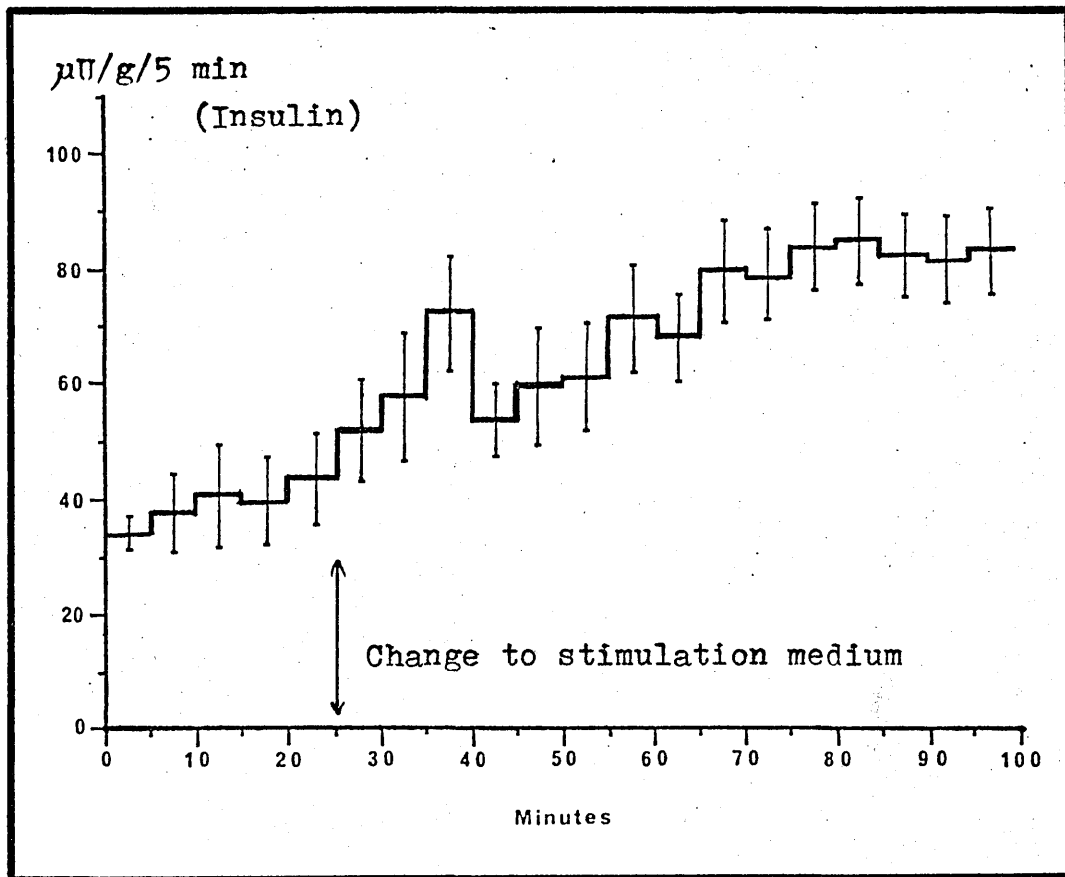
Figure 9.   Effect of phentolamine treatment during the last week
            of cold-acclimation on the basal and the glucose-
            stimulated insulin secretion rates. Values are the
            mean of six samples and the vertical bars represent
            the standard errors of the means in each case.

VIII.   Insulin secretion after bilateral adrenal demedullation
        of rats at the beginning of the last week of cold-
        acclimation

        Results are summarized in Table (6) and Fig. (10). Six

rats with an average body weight of 301.08 ± 4.38 g were used in

this set of experiments. Mortality rate after bilateral adrenal

demedullation was 8.3%. The average wet weights of the perfused

pancreases was 0.839 ± 0.014 g. The average perfusion rate was

2.127 ± 0.009 ml/5 min. and the perfusion pressure ranged between

85 and 94 mm Hg. Glucose concentrations in the basal medium and

in the stimulation medium were 4.135 mmol/l and 16.425 mmol/l

respectively.

        The basal rate of insulin release ranged between 14.92 ±

0.77 and 21.78 ± 2.98 µU/g/5 min. Insulin release in response to

glucose stimulation after the shift into the stimulation medium

perfusion exhibited the previously described biphasic pattern:

the first phase of insulin release peaked at 41.72 ± 2.79 µU/g/5 min;

the second phase of the biphasic response ranged between 25.05 ±

6.38 and 37.77 ± 5.67 µU/g/5 min. Both phases of insulin release

had their values significantly higher than those of the basal

secretion rate (p < 0.001). Insulin released in the first phase

was 13.3% of that released during the second phase for fifty minutes.

        Glucose uptake by the block of perfused tissue was 7.210 x

10^{-3} mmol/g/hour from the basal medium and 3.531 x 10^{-2} mmol/g/hour

from the stimulation medium.

Table 6: Data obtained from outflow of perfused
pancreases of cold-acclimated rats subjected to
bilateral adrenal demedullation performed at the
beginning of the last week of cold-acclimation

Sample	Volume ml	Glucose concentration mmol/l	Insulin concentration µU/ml	Insulin secretion rate µU/g/5 min.
1	2.16 ± 0.09	3.57 ± 0.13	8.28 ± 0.99	21.78 ± 2.98
2	2.15 ± 0.11	3.56 ± 0.13	7.97 ± 0.78	20.72 ± 2.31
3	2.14 ± 0.10	3.60 ± 0.14	5.80 ± 0.23	14.92 ± 0.77
4	2.10 ± 0.09	3.55 ± 0.12	7.40 ± 0.73	18.52 ± 1.59
5	2.12 ± 0.09	3.57 ± 0.13	6.82 ± 0.73	17.17 ± 1.27
6	2.16 ± 0.13	6.57 ± 0.33	10.62 ± 1.27	27.92 ± 4.16
7	2.14 ± 0.13	10.70 ± 0.45	16.17 ± 0.57	41.72 ± 2.79
8	2.12 ± 0.13	12.92 ± 0.19	14.25 ± 2.21	36.68 ± 6.71
9	2.11 ± 0.13	13.37 ± 0.18	11.82 ± 3.02	30.87 ± 8.82
10	2.09 ± 0.13	13.38 ± 0.15	9.18 ± 1.60	23.82 ± 4.96
11	2.11 ± 0.14	13.58 ± 0.20	9.62 ± 2.23	25.05 ± 6.38
12	2.12 ± 0.15	13.78 ± 0.27	10.22 ± 2.44	26.53 ± 6.92
13	2.12 ± 0.16	13.81 ± 0.24	11.38 ± 2.07	29.82 ± 6.19
14	2.13 ± 0.16	13.63 ± 0.17	11.48 ± 1.84	29.85 ± 5.55
15	2.13 ± 0.17	13.72 ± 0.18	12.98 ± 1.82	33.62 ± 5.76
16	2.13 ± 0.18	13.70 ± 0.18	13.12 ± 1.25	33.97 ± 4.83
17	2.17 ± 0.21	13.74 ± 0.16	14.30 ± 1.44	37.77 ± 5.67
18	2.13 ± 0.18	13.62 ± 0.16	12.58 ± 1.70	32.27 ± 5.56
19	2.10 ± 0.17	13.63 ± 0.15	12.32 ± 1.43	30.92 ± 4.45
20	2.11 ± 0.18	13.65 ± 0.14	13.22 ± 1.57	33.93 ± 5.70

i)   In all cases, n = 6
ii)   Mean body weight of animals = 301.08 ± 4.38 g
iii)   Mean wet weight of pancreas = 0.830 ± 0.014 g
iv)   All values are expressed as the mean and the standard error of
the mean.

uU/g/5 min (Insulin)

Change to stimulation medium

Minutes
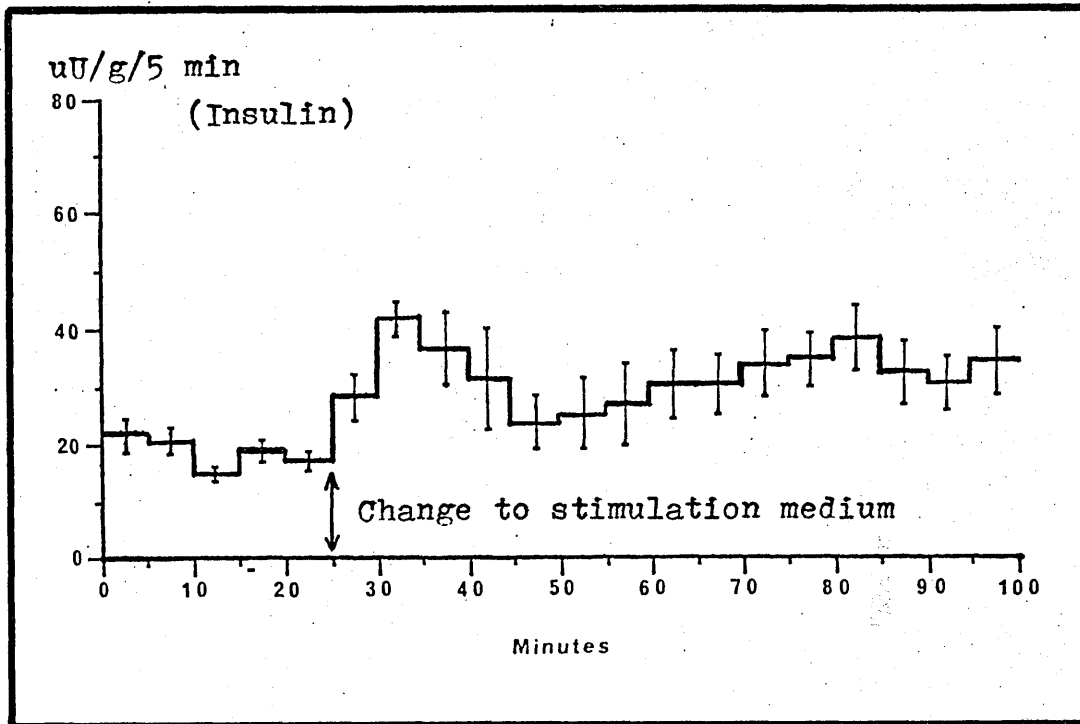
Figure 10. Effect of bilateral adrenal demedullation at the
beginning of the last week of cold-acclimation on
the basal and the glucose-stimulated insulin
secretion rates. Values are the means of six samples
and the vertical lines represent the standard errors
of the mean in each case.

Comparative light microscopic examination of sections of the normal adrenals and the demedullated adrenals were made and these showed complete removal of the medullary tissue in all the animals of the latter group. Photographs of a typical example of each group are shown in Fig. (11).

IX.   Summary of the statistical analyses of the results

Results of insulin secretion rates of the five different sets of experiments are summarized in Fig. (12), where point-to-point comparisons were made with the assistance of (t) values of the student t-test for matched paired samples, two-tailed test.

All values of insulin secretion rates before and during glucose stimulation in the main control set (MC) are significantly higher than all insulin secretion rates of the cold-acclimated (CA), the cold-acclimated with noradrenaline and phentolamine perfusion (CANP) and the cold-acclimated with adrenal demedullation (CADM) sets (all: $p < 0.001$); the insulin secretion rates of (MC) are significantly higher than insulin secretion rates of the cold-acclimated-phento-lamine treated (CAPT) set ($p < 0.01$).

Values of insulin secretion rates before and during glucose stimulation in the (CA) set are not significantly different from values of insulin secretion rates in (CANP) and (CADM) sets. But those same values of (CA) set, when compared with insulin secretion rates in the (CAPT) set, the level of difference prior to glucose stimulation is not significant, while during glucose stimulation,

values of insulin secretion rate in the (CAPT) set are significantly different (higher) from those of the (CA) set $(p < 0.02)$.

Values of insulin secretion rates in the (CAPT) set are significantly different (higher) from values of insulin secretion rates in the (CADM) set, prior to glucose stimulation $(p < 0.05)$, and during glucose stimulation $(p < 0.02)$.
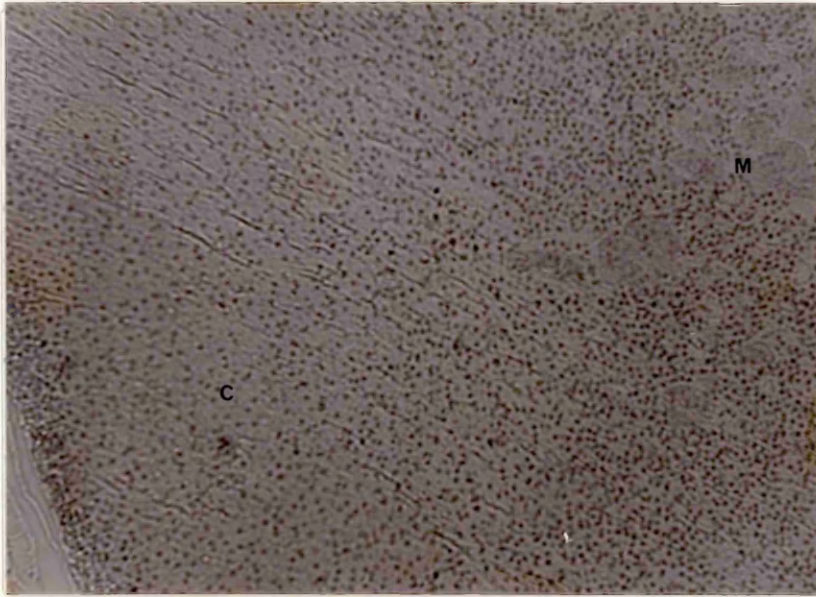
(a)
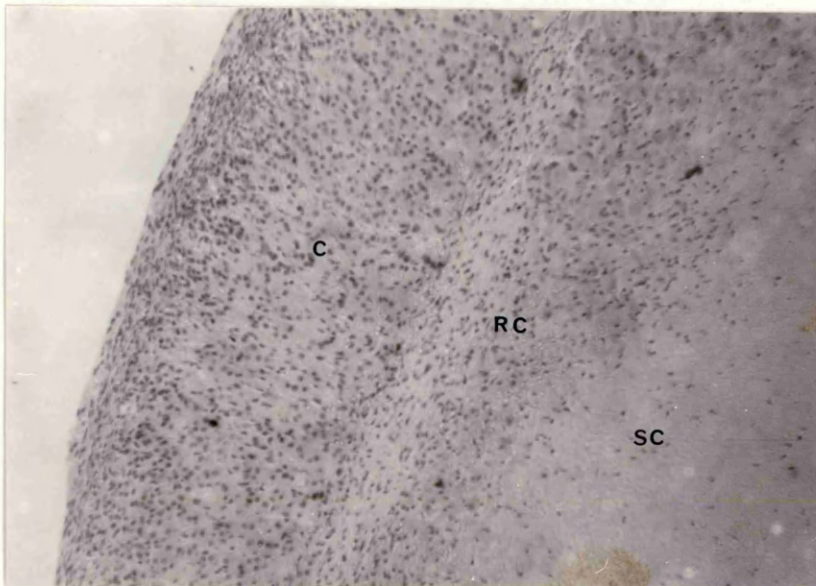
(b)

Figure 11.   Photomicrograph of adrenal gland (a) and demedullated
adrenal gland (b) of the rat.   Stained haematoxyline
eosine,100 X.   Key to labels: (c) cortical cells, (M)
medullary cells, (RC) regenerating cortical cells,
(SC) fibrous scar tissue.
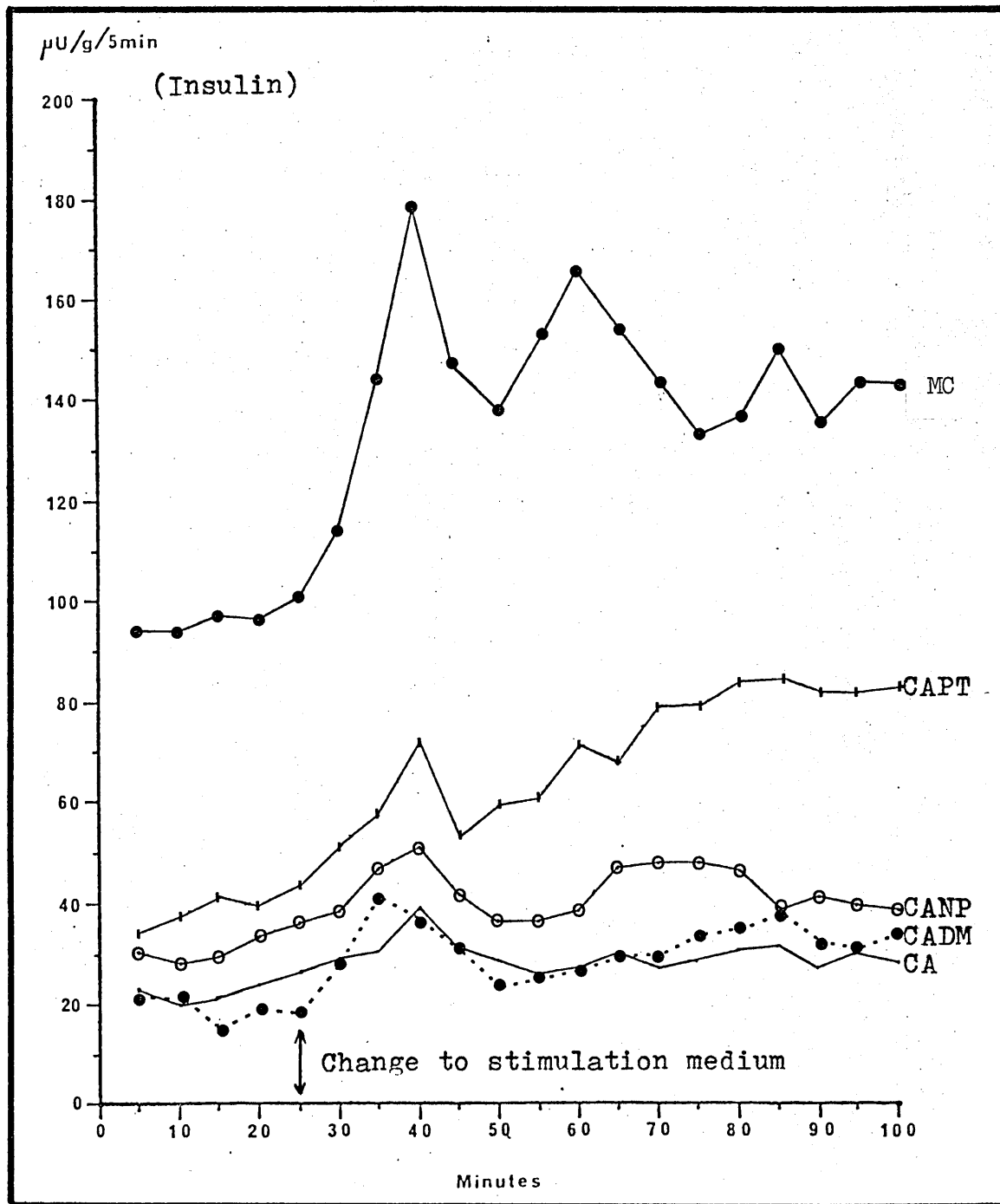
Figure 12. Comparative summary of basal and glucose stimulated insulin
secretion rates of the main control and the experimental
sets of rats: (MC) main control; (CAPT) cold-acclimated-
phentolamine treated; (CANP) cold-acclimated, noradrenaline
and phentolamine infused; (CADM) cold-acclimated, bilateral
adrenal demedullated; (CA) cold-acclimated.

DISCUSSION

I.   General observations

The physiology of cold exposure and cold-acclimation
has become a subject of great importance. It has been shown
that cold-acclimation alters basic metabolic interactions which
involve heat energy exchanges in the body of homeotherms. An
effect of cold-acclimation on the normal secretion range and
pattern of insulin from pancreatic islets has been proposed for
this investigation. Cold, as a stimulus, has been shown to increase
plasma noradrenaline content (Benedict, Fillenz and Stanford, 1977 a;
DesMarais and Dugal, 1951; Euler, 1971; Himms-Hagen, 1972;
LeBlanc and Nadeau, 1961; Nathanielsz, 1969; Sellers, Flattery,
Shum and Johnson, 1971; Shum, Johnson and Flattery 1969).
Furthermore, it has been demonstrated that an increase in cate-
cholamine levels may inhibit net or overall insulin secretion from
the pancreatic islets (Buse et al., 1970; Cerasi et al., 1969;
Malaisse et al., 1967 b; Yajima et al., 1977).

From the results of the present investigations it is
possible to make certain general observations with respect to the
effect of cold-acclimation on insulin secretion from the beta-
cells of the islets of Langerhans of the pancreas, as well as, the
regulatory influence of alpha-adrenergic receptors and sympatho-
mimetics on insulin secretion:

1.   Insulin secretion, from the isolated perfused pancreas, in response to a glucose stimulus exhibits a biphasic secretion pattern which is considered to involve storage compartments of insulin in the beta-cells of the pancreatic islets.

2.   Cold-acclimation reduces insulin secretion in the rat. This reduction might be a partial inhibition of insulin release in response to glucose stimulation and/or a suppression of insulin synthesis in the beta-cells.

3.   Insulin release from the isolated perfused pancreas is inhibited by the perfusion of high doses of noradren-aline.

4.   The reduction of insulin secretion is an alpha-adrenergic effect which is reversed by the action of the alpha-adrenergic antagonist, phentolamine.

5.   The regulatory effect of the alpha-adrenergic terminals in the beta-cells of the pancreatic islets is controlled directly by the sympathetic postganglionic fibres rather than by adrenal medullary secretion of catecholamines.

II.   <u>Discussion of the general observations</u>

1.   <u>The secretory dynamics of insulin:</u>

The insulin secretion response to glucose stimulation indicates that the insulin release is multiphasic. As the

glucose concentration of the perfusing medium reaching the

beta-cells is suddenly raised and kept at a high level (16 mmol/l),

the secretory response of these cells exhibits a biphasic

release of insulin. The same observation has been reported by

many workers (Basake et al., 1971; Curry et al., 1968 a;

Curry, 1971; Fussganger et al., 1969; Grodsky et al., 1963, 1970;

Grodsky, Bennett, Smith and Schmid, 1967; Hellerstrom, Andersson,

Gummarsson and Bern, 1974; Loubatières et al., 1970 a; Loubatières-

Mariani et al., 1973). Some evidence of a biphasic response to

glucose in vivo in man has been obtained by Cerasi and Luft (1967),

who measured insulin concentration in plasma after administration

of an intravenous glucose load.

In the present study, a peak of insulin release (179.44 ±

8.57 μU/g/5 min.) denoting the first phase, was observed after the

change to perfusion with the stimulatory glucose load. This first

phase Fig. (5) was followed by a low, but higher than basal

(94.34 ± 14.16 μU/g/5 min.) insulin output. The second phase of

insulin release was manifested as an overall increase in the rate

of insulin release and ranged between 133.99 and 166.43 μU/g/5 min.

This relatively high rate of hormone release was maintained for as

long as the glucose load is present and within the time limit of

the experiment.

The glucose load stimulus was intended to be a sharp square

wave of increase, but after glucose content analyses of the

collected samples it was shown that the total rise in glucose

concentration continued for a period of five to ten minutes.

This was because after the shift into the stimulation perfusion medium, the high glucose concentration underwent some dilution with the volume of the normal perfusion medium already filling the main perfusing head, the arterial cannula and the trunk of the coeliac axis. Thus, insulin release in the first phase was gradual for a short time but eventually reached the peak within five minutes once the glucose load was 8.325 mmol/l (150 mg/100 ml) or more. Curry (1971) showed a comparable effect on insulin release in response to a slow-rising glucose stimulus (30-300 mg/100 ml; duration 82 minutes) in the isolated perfused rat pancreas.

The multiphasic insulin secretion pattern probably indicates the existence of two pools of insulin (Curry et al., 1968 a,b; Grodsky et al., 1968; Porte and Pupo, 1969). Grodsky et al. (1970) have proposed a two-compartment model to account for the biphasic pattern of insulin release in response to glucose or tolbutamide stimulation. The essential feature of the model is the suggestion that only a small proportion (or compartment) that is, 2%, of pancreatic insulin is available for rapid release and that the remainder (the larger) compartment is released at a much slower rate. The initial rapid response lasts for some five minutes and is superimposed on a more protracted rise in the rate of insulin release over sixty minutes of perfusion. In the results presented here, the total amount of insulin released in the first phase was calculated to be about 10% of that released during the second phase over a period of fifty minutes. However, if the suggested superimposed initial rapid response of insulin release (which lasts for some five

minutes) is taken into consideration, then the net value of insulin released as such is calculated to be 1.8% of that released during the second prolonged phase.

The significance of the first phase of insulin release is obscure since it can be stimulated, in the absence of glucose, by sudden changes of concentrations of solutes in the perfusion medium, and that this first phase does not occur in response to a very slowly rising stimulus (glucose or otherwise) (Curry, 1971). Thus this first pool or compartment of insulin could serve as a safeguard against sudden increases in glucose concentration and it probably functions without a discriminatory mechanism for glucose.

The second phase of insulin release is glucose dependent and it could involve a second pool or compartment but it has not yet been ascertained whether the beta-cell receptors react primarily to the total amount of glucose which is presented, to the rate of change in plasma glucose or to a combination thereof (Curry, 1971). Nevertheless, there is a specific mechanism which results with insulin secretion being stimulated by a metabolite (Gagliandino, 1966) or possibly a product resulting from the metabolism of glucose which can also be supplied by other metabolizable sugars (Grodsky et al., 1963).

Glucose seems to stimulate insulin synthesis which is released in response to prolonged glucose stimulation during the second phase. In a set of four isolated perfused pancreas experiments (the results of which are not reported here) streptomycin and penicillin were included in the perfusion media at a final concentration of 100 U/ml each.

This resulted in a partial reduction of insulin release in
response to glucose stimulation throughout the second phase
of release, with no significant change in the first phase. This
could be interpreted as the result of inhibition of insulin
synthesis by the protein synthesis inhibitor streptomycin and
thus it could be concluded that the second pool or compartment
of insulin involves newly synthesised insulin as well as prestored
insulin, in contrast to the first pool or compartment which stores
insulin for immediate release. Gotfredsen (1976) included peni-
cillin and streptomycin (100 U/ml each) in the perfusion media of
the isolated perfused pancreas, but did not comment on the use
of those antibiotics especially in relation to the second phase of
insulin release. Curry et al. (1968 b) showed that puromycin had
no apparent effect on the first phase but there was a reduced out-
put of insulin in the second phase in response to glucose stimulation.

Glucose uptake by the perfused block of tissues was observed
to be in direct proportion to glucose concentration perfusing the
tissues. From the results of glucose uptake in this set of
experiments, the average amount of glucose taken up by the tissues
from the high glucose concentration media is 5.5 times that taken
up from the normal low glucose concentration media. These results
are in agreement with those of Snyder et al. (1970) who reported
that glucose uptake from 16.65 mmol/1 glucose medium was 4.8 times
more than the uptake from 3.33 mmol/1 glucose medium by isolated
rat islets.

This increased value of glucose uptake correlates with

the increased insulin release rate of the second phase, but it

is not certain whether this glucose is completely metabolised

before exerting its action as a stimulant for insulin release

and synthesis. Nor is it certain what proportion is responsible

for which mechanism. Furthermore, in the present study, the

glucose uptake values, together with arterio-venous oxygen tension

differences, served as reliable indicators of the viability of the

isolated perfused pancreas.

The precise sequence of events leading to the release of

insulin by the pancreatic beta-cell in response to glucose

stimulation remains an open question for further investigation.

2. Effect of cold-acclimation on insulin secretion

The insulin secretory response to glucose stimulation in

the isolate perfused pancreas of the cold-acclimated rat, showed

a very significant total reduction of insulin release in comparison

with the normal controls ($p < 0.001$) at all points. The biphasic

secretion pattern was still evident, Fig. (6). The basal insulin

secretion rate was about 23.3% of that in the normal; the peak

of the first phase in response to glucose stimulation was about

21.5% of the normal and the range of the second phase of release

was comparably reduced. There are a number of possible reasons for

the total reduction in insulin release in response to cold stress.

It could be either the result of reduced sensitivity of the beta-

cell to glucose stimulation or an overall reduction of compartment

size of both pools of insulin in the beta-cell, which, in other words, might mean a reduction in insulin synthesis.

Beck et al., (1967), Howland (1967) and Howland and Nowell (1968) demonstrated that rats exposed for (2-7) weeks to severe cold exhibited a significant decrease in serum immuno-assayable insulin levels. The fact that basal insulin release rate is reduced after cold-acclimation, supports the contention that there is an overall reduction of stored insulin and thus, a reduction in insulin synthesis. This total insulin reduction is accompanied by an increased insulin sensitivity in the cold-acclimated rat (Mansour and Hewitt, 1954; Depocas, 1961; Howland, 1967; Smith, 1962), and a decrease in pancreatic-extractable insulin (Baker and Ashworth, 1958) as well as, a reduction in the availability of insulin at the various centres of metabolic activity (Himms-Hagen, 1972). However, these observations do not rule out the possibility of a reduced response of the beta-cells to glucose stimulation which involves a very complicated mechanism influenced by many factors identified and characterized in terms of inhibition or enhancement of this glucose-induced insulin release. Little is known of their precise site of action in the beta-cell. It has been proposed that there are glucoreceptors, on the beta-cell, which involve two sites of mechanism: a substrate site and a regulatory site (Randle, Ashcroft and Gill, 1968). As

mentioned previously, the insulin secretory response to glucose is biphasic, but it is not clear whether the same glucoreceptor mechanism is involved in the two phases of the secretory response. The suggestion for the operation of the substrate site mechanism comes from the observation that sugars readily metabolized by mammalian tissues (e.g. glucose, mannose, and fructose) elicit insulin release, while non metabolizable sugars (e.g. galactose) do not stimulate release of insulin (Coore and Randle, 1964 a,b; Grodsky et al., 1963). The regulator site mechanism is still very hypothetical and needs further studies to establish the mechanism of this effect and its potential for a glucoreceptor mechanism.

During cold-acclimation the main reaction of many animals (including the rat) is a greatly increased secretion of noradrenaline (and adrenaline), as shown by many investigators (Basake et al., 1971; Benedict et al., 1977 a; Leduc, 1961 a,b; Nathanielsz, 1969; Roy, Sellers, Flattery and Sellers, 1977; Sellers et al., 1971; Shum et al., 1969). This enhanced secretion of catecholamines is of great importance in relation to insulin secretory mechanisms of the beta-cells of the pancreas, as well as in the overall process of cold-acclimation.

Having demonstrated that high noradrenaline concentrations inhibit insulin release in response to glucose stimulation (discussed in the next section) which is in accordance with the work of Porte and Pupo (1969), it might

be proposed that the reduction of insulin secretion after
cold-acclimation is due to the inhibitory effect of nor-
adrenaline released in response to the cold stress and that
this inhibition is acting on one or both effector mechanisms
of insulin secretion and synthesis. The mechanism of inhibition
of the insulin release as seen in this investigation will
therefore involve the alpha-adrenergic receptors of the pancreatic
beta-cells. Although it seems probable that this action involves
the inhibition of adenosine triphosphate (ATP) formation(Malaisse
and Malaisse-Lagae,1970 a), there could be an additional
inhibitory effect on calcium ion uptake which is suspected by
Hales and Milner (1968) and Hales (1970) to play an important
role in the process of insulin release. The sites of action of
other inhibitions may be glucose phosphorylation, calcium ion
flux and ATP formation, all of which are more or less related to
both mechanisms of insulin synthesis and release.

Although the synthesis of noradrenaline rises at the
beginning of the development of the cold-acclimation up to
four times the normal, a gradual decline takes place over
several weeks to a level about double the precold-exposure
value (Leduc, 1961 b; Sellers et al., 1971; Shum et al., 1969).
Nevertheless, the inhibitory influence on insulin release is
observed to be still effective after six or more weeks of
cold exposure, although a reduced sensitivity to nor-
adrenaline is reported to be prevailing by that time
(Raff, 1976).

The fact that noradrenaline suppresses insulin
secretion (and possibly synthesis) is very noteworthy
since this catecholamine, which is released from
sympathetic nerve endings, lying in close proximity to
beta-cells of the pancreatic islets (Lacy, 1957, 1967;
Shorr and Bloom, 1970), may be of greater significance
in modulating insulin release during the cold stress,
than is adrenaline which originates primarily from the
adrenal medulla and which was shown by many investigators
to increase during cold-acclimation as well (Depocas,
1960 a; Hammond and Hammolsky, 1970; Leduc, 1961 a,b;
Malaisse et al., 1967 b; Smith, 1962). It is therefore,
very probable that the influences of noradrenaline
(directly and indirectly) and adrenaline (indirectly) make
cold-acclimation possible in the presence of an insulin
deficiency, together with other metabolic changes and
mechanisms, such as increased mobilisation of free fatty
acids, reduced dependence on glucose as the main metabolite
and the involvement of new thermogenic centres such as brown
adipose tissue. The increased lipolysis in white fat, where
less inhibition of triglyceride lipase helps the process of
triglyceride hydrolysis and thus mobilization, all of which
require the presence of higher levels of catecholamines.
It has been demonstrated that some of the diabetic rats
undergoing prolonged cold exposure, do survive and exhibit

certain criteria of cold-acclimation (Poe, White, and
Davis, 1963), indicating that at least a "normal"
amount of insulin is not an absolute necessity for cold-
acclimation and suggesting that some compensatory
mechanism might be involved.

This altered steady state, achieved after cold-
acclimation in rats, which is characterised by increased
secretion of catecholamines and reduced availability and
secretion of insulin is very much similar to conditions
prevailing during exercise where there is suppression of
insulin secretion (Ahlborg, Felig, Hagenfeldt, Hendler
and Wahren, 1974; Ahrens, Bishop and Berdanier, 1972;
Berdanier and Moser, 1972; Harvey, Faloona and Unger, 1974;
Wright and Malaisse, 1968) as well as enhancement of cate-
cholamine secretion (Chin and Evonuk, 1971; Galbo, Holst
and Christensen, 1971; Haggendal, Hartley and Saltin, 1970;
Yajima et al., 1977). Both these effects contribute in the
mobilization and increase of glucose and free fatty acids
which are needed during exercise and also, comparably,
during cold stress.

3.   Effect of noradrenaline perfusion on insulin secretion

The perfusion of noradrenaline into the isolated perfused
rat pancreas resulted in an inhibition of insulin secretion in
response to glucose stimulation. This inhibition is dose-
dependent.

At low concentrations of noradrenaline, ranging

between (5 ng and 40 ng) per 100 ml of perfusion medium

the biphasic secretion pattern of insulin persisted,

although the insulin secretion rate fell with the increase

of noradrenaline concentration. At (80 ng and 100 ng) per

100 ml perfusion medium, the basal insulin secretion rate

was the same and constant Fig. (7), but the first phase of

insulin release in response to glucose stimulation was

completely abolished, which indicates that inhibition of

the first phase is an alpha-adrenergic phenomenon. Further-

more, the second phase of insulin release showed an overall

reduction in comparison with the control group $(p < 0.001)$,

but it exhibited a slight increase over the basal secretion

rate prior to glucose stimulation $(p < 0.05)$, which might

indicate a dual effect of alpha-adrenergic inhibition and a

probable second mechanism which over-rides the total in-

hibitory influence of noradrenaline.

It is well established that the adrenergic receptors

of the beta-cell of the pancreatic islets play an important

role in the regulation of insulin secretion, and it is accepted

that the activation of the alpha-adrenergic receptors result

in an inhibition of this secretion (Loubatières et al., 1967,

1970 a; Loubatières and Loubatières-Mariani, 1974; Malaisse

et al., 1967 b; Porte, 1967 a, 1969; Porte et al., 1966;

Sumi and Ui, 1975).

Porte and Williams (1966) demonstrated that nor-adrenaline has an inhibitory action upon glucose stimulated insulin secretion although it is considerably weaker in this action than is adrenaline. This difference in the degree of inhibition produced, may be due to the varying potency of these two catecholamines as alpha-adrenergic receptor stimulators (Day, 1975). Nevertheless, the perfusion of noradrenaline resulted in an inhibition of insulin release from the isolated perfused pancreas of normal rats which approached that inhibited level produced by the effect of cold-acclimation on rats. However, the main difference between the two situations is the presence of a first phase of insulin release in response to glucose stimulation in the isolated perfused pancreas of the cold-acclimated rat, whereas this first phase was abolished by the perfusion of high doses of noradrenaline in the presence of glucose load. This indicates a difference between the direct effect of exogenous noradrenaline on alpha-adrenergic receptors of the pancreatic beta-cells of normal rats and the effect resulting from gradual change during the process of cold-acclimation. During acclimation, noradrenaline secretion increases at the onset of the cold stress and subsides later but remains higher than the normal.

In the set of experiments with the isolated perfused pancreas (after cold-acclimation) where both the basal and the stimulatory perfusates included, simultaneously, noradrenaline and phentolamine (the alpha-adrenergic antagonist), the results

show that the pattern and amounts of insulin secretion
rates, compared with those of the cold-acclimated set,
exhibited no significant differences ($p \geq 0.5$). These
results indicate that there exists a distinct gradual
change of mechanism relating the regulatory effect of
adrenergic receptors with the net process of insulin
secretion from the beta-cells during the process of
cold-acclimation.

Malaisse et al., (1967 b) demonstrated that adrenaline
and noradrenaline inhibit insulin secretion from pieces
of rat pancreas incubated with glucose 8.325 mmol/l, and
suggested that this inhibitory effect is mediated through
alpha-receptors for it is blocked by alpha- but little
affected by beta- blocking agents. The same inhibitory
effect was demonstrated in man (Porte et al., 1966), in
dog (Porte, Girardier, Seydoux, Kanazawa and Posternak,
1973), in rat (Loubatières-Mariani, Chapal, Ribes and
Loubatières, 1977), and in rabbit (Coore and Randle, 1964).

Buse et al., (1970) reported that endogenous cate-
cholamines have an inhibitory action upon glucose induced
insulin secretion in man; yet in many patients with elevated
plasma levels of catecholamines (Vance, Buchanan, O'Hara,
Williams and Porte, 1969) and in those in metabolic stress
states in which catecholamine levels may be expected to be
increased, basal insulin concentrations are either normal
or increased. The explanation for this finding is probably

related to the remark that long-term steady state

responses to glucose are not inhibited by adrenaline

and noradrenaline. Such observations and studies

support the previous suggestion that basal insulin con-

centrations are secreted from a pool that is functionally

distinct from the storage pool(s) for glucose stimulated

insulin responses.

Finally, there exist some discrepancies in the

response of beta-cells to their activation by different

adrenergic stimulating agents, observed not only between

the different animal species but also within the same

species (Loubatières-Mariani et al., 1977), depending on

the age of the animals under experiment. Thus all

experimental animals in this investigation were chosen from

the same age group in order to eliminate such inconsistancies.

Similarly, each whole set of experiments was performed during

a period of ten to fifteen days in order to avoid any

possibility of effect of seasonal variation on noradrenaline

plasma levels, or to noradrenaline sensitivity (Leduc,

1961 a,b).

4.   Effect of phentolamine treatment during cold-acclimation

Treatment of the rats during the last week of cold-

acclimation with subcutaneous injections of phentolamine

(the alpha-adrenergic antagonist) resulted in a pronounced

elevation of insulin secretion compared with that of the

cold-acclimated set, in response to glucose stimulation, ($p < 0.02$).
But, this increase did not restore the insulin secretion
rates up to the values of the normal control set.

The basal insulin secretion rates after phentolamine
treatment are not statistically different from the basal
rates of insulin release exhibited in the cold-acclimated
set and this indicates that basal insulin, although reduced
after cold-acclimation, is not strictly modulated by endo-
genous catecholamines. Thus it is not influenced by the alpha-
adrenergic action of phentolamine on catecholamines and in
particular noradrenaline, which supposedly inhibits insulin
secretion as a result of cold-acclimation. It is therefore
suspected that there exists a distinct mechanism that
regulates basal insulin release (and possibly synthesis),
and that it might involve a different storage pool of insulin
in the beta-cells. Such a finding supports the same hypothesis
of the existance of insulin storage pools discussed earlier
(Grodsky, 1972 a,b; Porte and Pupo, 1969).

In response to glucose stimulation, the pattern of
insulin release in this set of experiments is biphasic,
Fig. (9). The first phase peak is significantly greater than
that of the cold-acclimated set ($p < 0.02$), and at the same
time it is significantly less than the peak value of the first
phase of the normal control set ($p < 0.001$). Similarly, the
second phase of insulin release has all its values significantly

higher than those of the second phase values of the cold-acclimated set (p < 0.01) and at the same time they are significantly lower than those of the normal control group (p < 0.01).

From these results, and in view of the report by Malaisse et al., (1967 b) and Porte (1967 b), it is evident that phentolamine blocked the inhibitory effect of nor-adrenaline upon insulin secretion in response to glucose stimulation. Similar actions of alpha-adrenergic blockers during exercise are reported by Harvey et al. (1974). These results support the hypothesis that insulin release in response to glucose stimulation involves pools or compart-ments different from those involved in the basal release of insulin. Porte (1969) demonstrated that alpha-adrenergic blocking agents can suppress the inhibitory effect of endogenously secreted catecholamines and thus Bressler et al., (1968) and Buse et al., (1970) suggested that it may seem logical that in vivo the alpha-adrenergic blocking agents tend to increase the basal or the glucose-stimulated levels of circulating insulin. Buse et al. (1970) showed that pre-treatment of normal subjects with phentolamine resulted in a significantly greater insulin response in comparison with controls, the mean plasma insulin levels being 38% greater in the experimental group, five minutes after glucose injection. Despite this greater insulin response, the blood glucose levels were similar in these two groups suggesting that counter-regulatory mechanisms may have been brought into action by the increased insulin release during alpha-blockade.

Adrenaline retains its glycogenolytic actions during alpha-
blockade (Porte, 1967 a) and may also stimulate glucagon
secretion (Gerish, Karam and Forsham, 1973; Gerish, Langlois,
Noacco, Schneider and Forsham, 1974).

Such studies as well as the work by Robertson and
Porte (1974) support the conclusion that endogenous cate-
cholamines modulate the insulin secretory response to
glucose stimulation. Similarly, Potter et al., (1977)
reported that alpha-adrenergic agonists inhibit insulin
release, a finding which agrees with the above mentioned
studies that demonstrated phentolamine to be without effect
on basal insulin levels when given alone, suggesting that
basal insulin secretion is not modulated by endogenous alpha-
adrenergic activity. This is also in agreement with the
results of the present investigation, where exogenous alpha-
adrenergic blockade did not have an effect on basal insulin
secretion. Furthermore, Efendic, Cerasi and Luft (1973) and
Imura, Kato, Ikeda, Morimoto and Yawata (1971) found no effect
of phentolamine upon basal insulin levels. Udenfriend (1968)
reported that alpha-adrenergic blockage resulted in more cate-
cholamines being synthesized in sympathetically innervated
tissues than during exercise or exposure to cold and explained
these observed effects of phentolamine postulating that the
resulting hypotension influences the baroreceptors which activate
the appropriate centers in the central nervous system to
increase sympathetic activity. It is this increased

sympathetic nerve activity (in the presence of receptor blockade) which liberates noradrenaline (and adrenaline) and thereby brings about a simultaneous increase in their synthesis. Such an increase in noradrenaline synthesis could very well have happened in the phento-lamine treated rats in this set of experiments but the overall effect did not show an inhibitory influence, and instead, the insulin secretory response to glucose stimulation increased.

5.   Alpha-adrenergic regulation of insulin secretion

It is established that the process of cold-acclimation in rats results in a reduction of insulin secretion, both on the basal secretion and on the response to glucose-stimulation, and that this phenomenon is mediated through the effect of catecholamines (mainly noradrenaline). Many workers have demonstrated this inhibitory effect of the catecholamines upon insulin secretion, and have indicated that adrenaline is a more potent inhibitor of insulin secretion than noradrenaline. Furthermore, it is well established that the process of cold-acclimation increases endogenous catecholamine synthesis, both in the adrenal medulla (adrenaline and noradrenaline) and in the adrenergic nerve endings (noradrenaline).

So in order to verify the hypothesis that was put forward that this reduction of insulin secretion is brought into effect by the action of noradrenaline and that it is

mediated through the sympathetic nerve fibres, the effect on insulin secretion of bilateral adrenal demedullation, during the last week of cold-acclimation was studied. The results show that there are no significant differences in insulin secretion rates between this studied set of experiments and the cold-acclimated set ($p \geq 0.50$). Basal insulin secretion rates in both sets were almost identical Fig. (12). Both experimental sets exhibited the biphasic insulin secretion pattern in response to glucose stimulation, peak values of the first phase of insulin release were identical, as well as both ranges of the second phase. These results clearly demonstrate that catecholamines originating from the adrenal medulla were not responsible for the inhibitory effect on insulin secretion and/or synthesis in the beta-cells of the pancreatic islets.

Leblanc and Nadeau (1961) demonstrated that after adrenalectomy the quantity of noradrenaline in the urine remained essentially unchanged in both the adrenalectomized and the normal rats. This indicates that noradrenaline secretion occurs outside the adrenals. Similarly, Hsieh, Carlson and Gray (1957) demonstrated that urinary noradrenaline is from extra-adrenal origin in adrenalectomized rats. In this context, it is also pertinent to recall that in demedullated animals, the urinary excretion of noradrenaline has been found to be elevated at rest and its increase following cold exposure was greater than in

controls (Johnson et al., 1966). Furthermore, Leduc
(1961 a,b) reported that increase of adrenaline excretion
induced by cold exposure was mainly due to an increased
secretion from the adrenal medulla whilst the increased
noradrenaline output resulted from an increased release
from the adrenergic nerve endings.

Thus it might be concluded that noradrenaline
originating from sympathetic postganglionic nerve endings
is the effective catecholamine which inhibits insulin
secretion during cold-acclimation. Phentolamine treatment
during the last week of cold-acclimation which resulted in
an increased insulin secretion in response to glucose
stimulation, supports this conclusion. Phentolamine
blocked the inhibitory effect of noradrenaline on insulin
secretion. This blockade was very specific in action and
it involved only the mechanism of insulin release in response
to glucose stimulation and did not alter the basal insulin
secretion rate. This basal insulin might be coming from a
distinct pool with a specific release mechanism. It seems
therefore that the regulatory influence of the sympathetic
nervous system on the beta-cells is closely associated with
glucose stimulation and involves a related insulin storage
pool, although the hyperactivity of the alpha-adrenergic
terminals associated with the beta-cells modulate the
general synthetic and release mechanism of insulin during
cold-acclimation. The total reduction in insulin secretion

rates observed during cold-acclimation is, thus, controlled directly by the sympathetic postganglionic fibers rather than by adrenal medullary secretions of catecholamines. Roy et al., (1977) suggested that the increased excretion of noradrenaline, adrenaline and metabolites is indicative of increased sympathetic activity and because of its extent, is highly suggestive of increased synthesis of catecholamines. Nevertheless, adrenaline could play as important a role as noradrenaline, but only after exhaustion or failure of the noradrenaline reserves. It was shown that after a drug-induced decrease of noradrenaline, adrenaline excretion increases, which indicates that the two hormones can replace each other in their thermogenic as well as other effects (Jansky, 1973). Besides all the above evidence, some specific laboratory studies on rats have shown that the catecholamines in the adrenal venous effluents after cold-acclimation are largely composed of noradrenaline and that the total output of catecholamines greatly exceeds control values (Smith, 1962). Such an increase in total cate-cholamines due to cold-acclimation could be of vital importance in activating the proper homeostatic mechanisms involved in the process of cold-acclimation. They might concern other target organs that have to do with various metabolic and thermogenic processes other than the beta-cells of the Islets of Langerhans of the pancreas, such as the liver, the muscles, the brown adipose tissue and the circulatory system.

Hart (1963) pointed out that in cold-acclimated
rats, the control of cold thermogenesis shifts during
cold-acclimation to the sympathetic autonomic system.
The present results of the bilateral adrenal demedullation
experiments exclude the effect of adrenal catecholamines on the
beta-cells. These results indicate a direct effect of neural
input upon insulin secretion. In order to demonstrate this
direct neural response, an attempt was made to study the
effect of sympathetic postganglionic stimulation on insulin
release. However this proved to be unfruitful because of
the difficulty in locating and tracing the fine postganglionic
fibres to the rat pancreas. Porte et al. (1973) were able to
demonstrate the direct sympathetic nervous effect on insulin
release in the pancreas of the dog. Similarly, stimulation
of the splanchnic nerve (in the calf) inhibits glucose
induced insulin release (Bloom et al., 1973). Furthermore,
pancreatic sympathectomy in rats, as reported by Sacca et al.,
(1977), resulted in a severe hypoglycemia due to increased
insulin release. However, this was only a functional pancreatic
sympathectomy which they defined as adrenal demedullation with
intraperitoneal reserpine injection (2 mg/Kgm).

Fluorescence microscopy has been used to demonstrate
adrenergic nerve fibres in islets of several mammalian species
(Cegrell, 1963) and in the rat (Woods and Porte, 1974).
Further evidence suggesting that the autonomic nervous system
may be involved in the fine regulation of insulin secretion is

the demonstration of increased insulin secretion following
vagal stimulation (Frohman et al., 1966, 1967; Kaneto et al.,
1967).

In a brief summary of the mechanism associated with
the adrenergic regulation of insulin secretion, it is
pertinent to mention that the beta-cells of the pancreatic
islets are equipped with an adenyl cyclase-cyclic AMP-
phosphodiesterase system. Turtle and Kipnis (1967 a)
reported that alpha- and beta- adrenergic stimulation
respectively decrease and increase the cyclic AMP content
of the pancreatic beta-cells in vitro. Catecholamines are
considered to affect the activity of this system (Porte
and Robertson, 1973). In this connection it is argued that
although beta-stimulation could increase insulin secretion
via an accumulation of cyclic AMP within the beta-cell, alpha-
stimulation inhibition of insulin secretion occurs via a
mechanism independent of any action it may have to reduce
the cyclic AMP concentration. It is proposed that alpha-
adrenergic inhibition of insulin secretion may be mediated
by a reduction of islet calcium ion uptake as demonstrated by
Malaisse et al., (1970). Adrenaline (and noradrenaline) which
inhibit glucose-induced insulin secretion (Milner and Hales,
1968, 1969), also inhibited glucose-induced ($^{45}Ca^{++}$) uptake
(Malaisse-Lagae & Malaisse, 1971), while insulinotropic
agents were found to stimulate calcium uptake. Cyclic AMP

concentrations within the beta-cell are then seen as being maintained by a beta-adrenergic mechanism, whilst an alpha-adrenergic mechanism prevents the complete expression of this effect upon insulin secretion, possibly by inhibiting the calcium ion accumulation within the beta-cell. Although the potentiation of glucose-stimulated insulin release by cyclic AMP is specific regarding the adenosine moeity (Lernmark, 1974), the mechanism by which the adenylcyclase system affects insulin release is not fully understood, but it has been suggested that cyclic 3',5'-AMP could activate glycogen phosphorylase in beta-cells (Malaisse et al., 1967 c). This suggestion is based on the following observations: pancreatic tissue from normoglycemic rats, containing no detectable glycogen in the islet tissue, does not secrete insulin in the absence of glucose, and the stimulant effects of glucose and theophylline are reduced or abolished by mannoheptulose and 2-deoxyglucose. Furthermore, tissue obtained from rats made hyperglycemic with infusion of glucose, and containing glycogen within the islet tissue, responds to theophylline even in the absence of glucose. This stimulant effect is inhibited by 2-deoxyglucose but not by mannoheptulose, a result that suggests that the glucose normally used by the isolated pancreatic tissue is extracellular. In tissue from hyperglycemic rats, theophylline could stimulate glycogen phosphorylase and so provide an intracellular source

of glucose from stored glycogen. But since no such glycogen store is present in normal islets, this could not be the only operative mechanism.

The present results suggest that the alpha-adrenergic terminals of the sympathetic nervous fibres may be intimately involved in the mechanism, which control insulin secretion in the normal and the cold-acclimated rat pancreas. Stimulation of the sympathetic system and the release of catecholamines particularly noradrenaline, at adrenergic nerve terminals, are characteristics of cold stress. The high rate of loss of heat due to cold, necessitates a hyper active thermogenic process which utilizes larger quantities of glucose and free fatty acids than normal. This increased need for nutrients is supplied by the action of the catecholamines on the liver and the peripheral depots of fat, and thus thermogenic centres are able to use efficiently either glucose or fatty acids, without any change in plasma insulin concentration (which is, evidently, low during cold-acclimation). However, should the level of insulin increase for any reason, the release of these nutrients from the liver and fat cells would be impared as insulin suppresses both the release of glucose from the liver (Franckson, Arnould, Malaisse and Conrad, 1964; Larner, Villar-Palasi and Richman, 1959; Norman, Pierguilio, Reid, Lester and Hechter, 1959), and of free fatty acids from fat depots (Halperin & Denton, 1969; Jungas and Ball, 1963, 1964; Mahler, Stafford, Tarrant and Ashmore, 1964; Toomey, Shaw, Reid and Young, 1970). This antilipolytic effect of insulin

on adipose tissue is, undoubtedly, one of the most

important physiological actions of this hormone. That

anti lipolysis is not simply due to reesterification of

newly hydrolysed triglyceride fatty acids comes from

the observation that the release of glycerol, as well as free

fatty acid release, is decreased by the presence of insulin

(Jungas and Ball, 1963, 1964). Since triglycerides are

usually hydrolyzed to glycerol and free fatty acids within

the adipocyte (Butcher, 1966), (and the fatty acids can be

reesterified), glycerol release gives a more accurate

reflection of the rates of lipolysis than does free fatty

acid release (Chlouverakis, 1967). Insulin may inhibit the

release of free fatty acids from adipose tissue by two

mechanisms: a direct antilipolytic effect not dependent

on glucose, and one that promotes intracellular reesteri-

fication of free fatty acids via the production of increased

amounts of alpha-glycerol phosphate, secondary to augmented

glucose metabolism (Blecher, 1968). Although not in complete

agreement (Lisch et al., 1973; Mahler et al., 1964), most

workers have found that insulin also inhibits basal lipolysis,

i.e., the rate of glycerol release observed in the absence of

added lipolytic hormones (Chlouverakis, 1967; Fain, 1967;

Fain, Kovacev and Scow, 1966); The important role of insulin

as an antilipolytic agent is further illustrated by the

observation that lipolysis appears to be sensitive to very

low levels of insulin (Fain et al., 1966). It is pertinent

to mention here that a large number of hormones are known to stimulate the release of free fatty acids from adipose tissue. These include adrenaline, noradrenaline, glucagon, ACTH, TSH, glucocorticoids, and growth hormone.

Thus, with all the preceding arguments and experimental results put together, it might be concluded that during cold-acclimation, by inhibiting insulin secretion, the catechol-amines (especially noradrenaline released at the sympathetic nerve endings) are better able to sustain an altered meta-bolic steady state, and to maintain an adequate supply of nutrients for general body metabolism.

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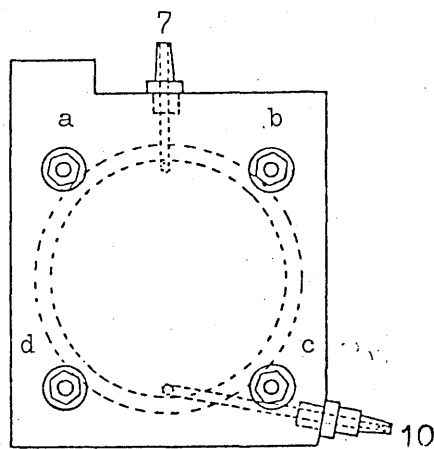   environment. Oecologia-Allemanica, 29(1), 11-26.
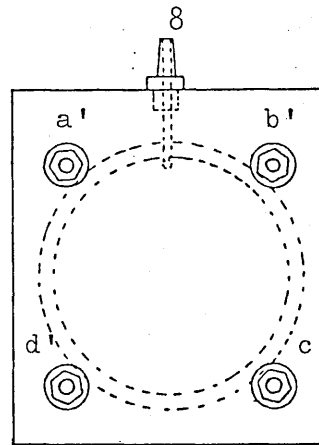
APPENDICES

Schematic diagram of the screen-cylinder oxygenator
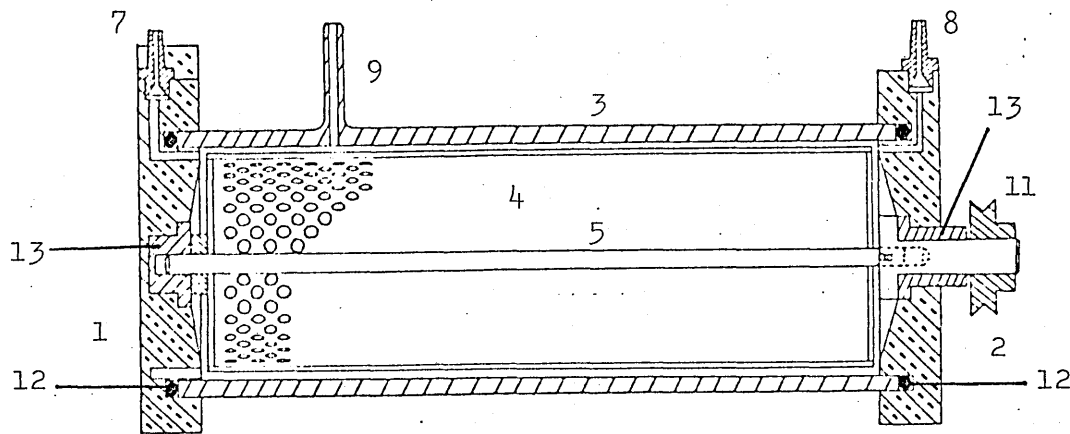
Side View

end plate 1   end plate 2

Key to numbers:

1 – Perspex end plate 1.
2 – "   "   end plate 2.
3 – "   "   cylinder jacket.
4 – Screen cylinder, pore size:
    3 mm diameter.
5 – Stainless steel shaft.
6 – aa', bb', cc', dd': four
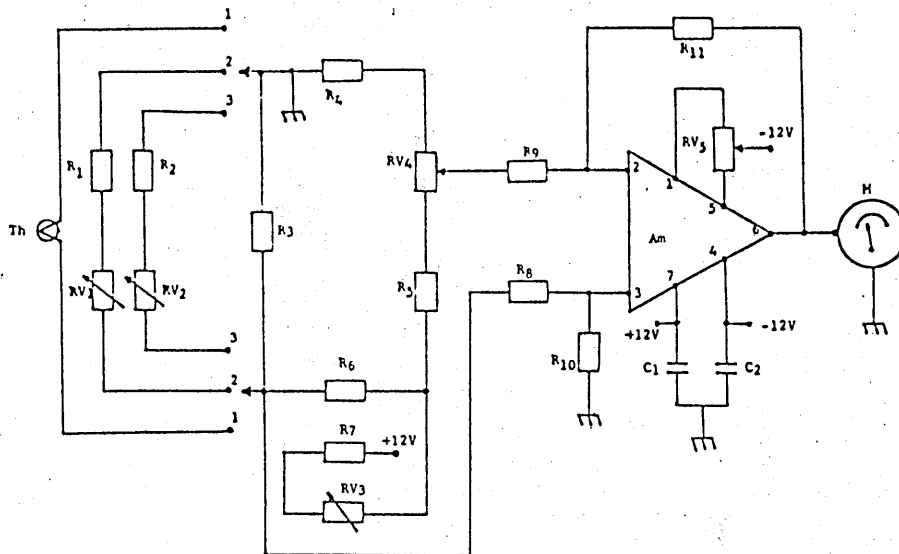   metal nuts placed at the
    ends of threaded rods.

7 – Gas inlet.
8 – Gas outlet.
9 – Perfusate inlet.
10 – Perfusate outlet.
11 – Brass pulley.
12 – Rubber O-ring seals.
13 – Stainless steel bearing,
     of the shaft.

Scale 1:2.26

Temperature Meter Unit

Components list:

$R_{1,3}$ = 1K2, $\frac{1}{2}$W, 2%, metal oxide.

R_2 = 820, " " " "

$R_{4,5}$ = 470, " " " "

R_6 = 560, " " " "

R_7 = 1K5, " " " "

$R_{8,9}$ = 10KO, " " " "

$R_{10,11}$ = 27KO " " " "

$RV_{1,4}$ = 200, Lin. carbon

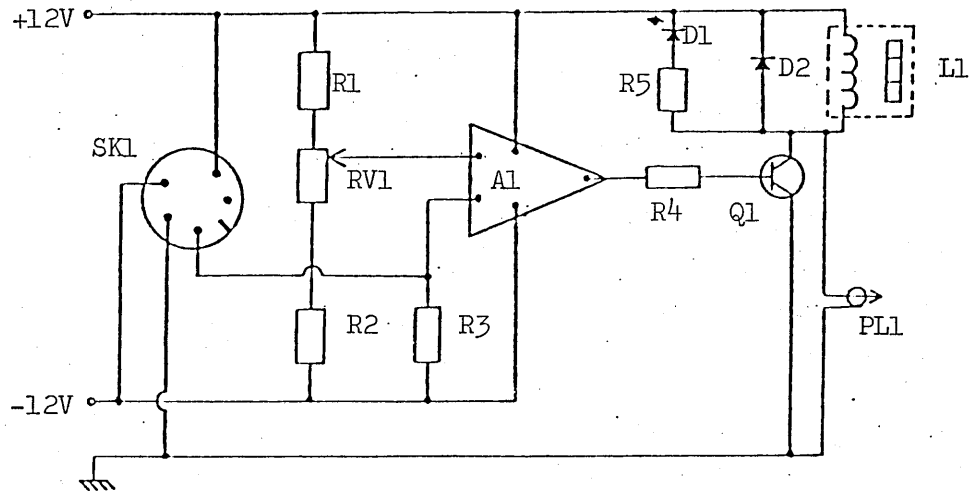RV_2 = 500, " "

RV_3 = 5KO, " "

RV_5 = 10KO, " "

$C_{1,2}$ = 0.01Z

Th=Glass bead thermister
    RS-151-029.
Am=Amplifier,4131T,R.S.
M =Ammeter(100µ amps) to
    read 30-40 C in 0.1 units.
Case 2 piece metal case.

Appendix 3

Drop Counter Control Unit

Components list:

D1	Min LED indicator, red.
D2	1N4148.
A1	741 op Amp.
Q1	2N3053 s.l., NPN.
R1,2	2K7, $\frac{1}{2}$w, 2%, metal oxide.
R3	3K9, $\frac{1}{2}$w, 2%, metal oxide.
R4	5K6, $\frac{1}{2}$w, 2%, metal oxide.
R5	1K0, $\frac{1}{2}$w, 2%, metal oxide.
RV1	5K0, Lin. carbon.
L1	Veeder Root K.J. 1643.
SK1	DIN 5-240 socket.
PL1	3.5 mm Jack Plug.
Case	2 piece metal case.

Appendix 4

Oxygen Dissociation Curve of FX-80

This section presents the details of the oxygen dissociation curve of FX-80 (Perfluoro-2-butyl tetrahydrofuran, ICN Pharmaceuticals Inc.) suspended in Krebs-Henseleit bicarbonate medium by ultrasonic disintegration. The FX-80 suspensions were equilibrated with different oxygen percentages in gas mixtures with constant $P(CO_2) = 35.65$ mmHg, for 20 min. each at a fixed temperature $37^{\circ}C$. Analyses of total oxygen content of 2 ml samples were done by the Van Slyke method. Percentage saturations of oxygen, at the different partial pressures of oxygen used, were calculated and listed in the following table:

Sample	% O_2 in gas phase	$P(O_2)$ in gas phase	ml O_2/2 ml perfusate	ml O_2/100 ml perfusate at s.t.p.
A	2.2	15.5	0.17	7.7
B	3.8	26.7	0.64	28.9
C	5.4	38.0	0.82	37.0
D	6.6	46.4	0.95	42.8
E	8.3	58.3	1.06	47.8
F	21.8	153.2	1.12	50.5
G	40.3	283.2	1.12	50.5