Some Biochemical Studies on the Islets of Langerhans

(Factors influencing Secretion of Insulin)

(SUMMARY)

THESIS SUBMITTED TO THE ALIGARH MUSLIM UNIVERSITY, ALIGARH, FOR THE DEGREE OF Doctor of Philosophy IN Biochemistry

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The endocrine cells have a unique property of being able to respond to a specific physiological stimulus with a resultant release of stored hormone in a modulated fashion. The mechanism through which extracellular factors govern the intracellular events in the endocrine pancreas remain poorly defined. The secretion of insulin by pancreatic B-cells is stimulated by sugars, amino acids, ketone bodies, hormones, cAMP and its derivatives. Insulin release is the result of a series of phenomenon including synthesis of proinsulin in the endoplasmic reticulum, transport of proinsulin to the Golgi apparatus, formation and maturation of granules, conversion of proinsulin to insulin, transfer or conversion of granules to a labile form and finally release of insulin from the B-cells. Lectins from Agaricus bisporus and Agaricus campestris stimulate insulin release from islets of Langerhans. Binding of A. bisporus PHA-B stimulated insulin release were inhibited in parallel by a glycopeptide containing the oligosaccharide receptor for lectin, thus lectin binding appears to be essential for the expression
of its insulin releasing activity. The studies have been
carried out with the view to obtain some information regarding
the effect of some extracellular events on the mechanism of
insulin release. Lectin PHA-B and streptozotocin have been
used for our studies on their interaction with their possible
receptors on B-cells membranes.

ISOLATION, PURIFICATION, AND CHARACTERIZATION OF LECTINS
FROM AGARICUS BISPORUS

The need for the development of a more efficient and
simple method for the purification of Agaricus bisporus lectin
was felt especially when one of its fraction PHA-B was found
to be able to release insulin from the islets of Langerhans.

The present purification procedure consists of Con A-
sepharose affinity chromatography and phosphocellulose chro-
matography. A. bisporus PHA was purified with a recovery
of 90.8% and 6 fold purification by Con A -sepharose column
chromatography when crude extract (NaCl extract and heat
treated) was passed through Con A-sepharose column and was
eluted with 0.1 M mannose in phosphate buffered saline, 0.02 M,
PH 7.2. The preparation was resolved into two hemagglutinating
fractions by phosphocellulose column chromatography thus about
80% of the hemagglutinating activity of the Con A-sepharose
eluate was eluted at pH 5.85 (fraction PHA-A), whereas 15% of the hemagglutinating activity was released from the column when the pH of the eluting buffer was raised to 8.2.

The Con A-sepharose eluate exhibited one major and one minor band on polyacrylamide gel electrophoresis corresponding to PHA-A and PHA-B. The PHA-A and PHA-B obtained by phosphocellulose column chromatography exhibited single band separately on polyacrylamide gel electrophoresis, showing homogeneous preparation of the two PHAs fractions.

The molecular weights of the purified \textit{A. bisporus} PHA-A and PHA-B as determined by sephadex G-100 gel filtration were 64,100 and 58,000 respectively. The neutral sugars content of the purified PHA was found to be 2.5% as estimated by phenol sulphuric acid method.

**EFFECT OF AGARICUS BISPORUS PHA-B ON GLUCOSE METABOLISM IN ISOLATED ISLETS OF LANGERHANS**

These studies were carried out in order to study the effect of \textit{A. bisporus} PHA-B on oxygen uptake by the isolated islets of Langerhans. It was found that in the presence of 2 mM glucose alone and 2 mM glucose + 17.5 \textmu g/ml \textit{A. bisporus} PHA-B (that produces half maximal stimulation of insulin release) there was no significant difference in the \textmu l of O$_2$ uptake.
Similarly no significant change in $O_2$ uptake was observed when glucose concentration was raised to 20 mM and keeping PHA-B concentration $17.5 \mu g/ml$. Thus it appeared that the $A.\ bisporus$ PHA-B did not alter the rate of islet glucose metabolism.

**IN VITRO STUDIES ON AGARICUS BISPORUS PHA-B MEDIATED INSULIN RELEASE FROM ISLETS OF LANGERHANS**

The study was carried out in order to determine the insulin secreting property of the purified $A.\ bisporus$ PHA-B. For in vitro studies islets were incubated with $A.\ bisporus$ PHA-B ($17.5 \mu g-100 \mu g/ml$) in the presence of 2 mM glucose in KRB medium. Samples were drawn at an interval of one hour and the insulin content was estimated by radioimmunoassay.

It was found that at 2 mM glucose + $17.5 \mu g/ml$ PHA-B the insulin release was $109 \pm 4 \mu U/5$ islets/hr as compared to the control (2 mM glucose), which was $33 \pm 5 \mu U/5$ islets/hr. Thus, there was 3.3 fold increase in insulin release in response to the lectin as compared to the control. The stimulation of the insulin release was found to be lectin dose as well as time dependent. The maximal stimulation of the insulin release was obtained at lectin concentration of about $60 \mu g/ml$ after which the release of insulin tends to be stationary. The lectins mediated insulin release from islets of Langerhans proceeded as a linear function of time for about one hour.
The insulin release in presence of 2 mM glucose alone and 2 mM glucose + 17.5 μg/ml (A. bisporus PHA-B) was enhanced from the beginning and after 20 minutes of incubation the release in the presence of A. bisporus PHA-B was about three times as compared to 2 mM glucose alone and the same proportion was maintained throughout the one hour period of incubation.

It is conceivable, therefore, that lectin induces alterations in the structure or arrangement of specific membrane components may in some way facilitate the process of exocytosis with resulting acceleration of insulin release.

45Ca2+ UPTAKE STUDIES IN THE PRESENCE OF GLUCOSE AND A. BISPORUS PHA-B BY ISOLATED ISLETS OF LANGERHANS

D-glucose alters the state of Ca2+ in islets of Langerhans and enhances the rate of uptake of isotope. It has been assumed that B-cells are responsible for observed stimulatory effect on islet calcium uptake and a very strict correlation between islet radioactivity and insulin release has been demonstrated. These studies were carried out in order to find out the ability of the purified A. bisporus to enhance 45Ca2+ uptake by islets as A. bisporus PHA-B is also an insulin secretagogue.

It was found that in the presence of 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B the 45Ca2+ uptake by islets was enhanced more than eleven folds as compared to the control.
(2 mM glucose) whereas when the lectin concentration was raised to 50 μg/ml, the corresponding $^{45}\text{Ca}^{2+}$ uptake by islets was increased more than 30 folds as compared to the control. The $^{45}\text{Ca}^{2+}$ uptake was found to be lectin dose and time dependent. The rate of the $^{45}\text{Ca}^{2+}$ uptake increased with the concentration of the lectin and the maximal uptake was achieved at lectin concentration of about 60 μg/ml and then the rate slows down until it reached a stationary phase at about 75 μg/ml lectin concentration. Thus there was a strict correlation between insulin release and $^{45}\text{Ca}^{2+}$ uptake by islets. The maximal insulin release as well as maximal $^{45}\text{Ca}^{2+}$ uptake was achieved at lectin concentration of about 60 μg/ml. Both the studies were carried out in the same set of experiment.

For time course studies islets were incubated with 2 mM glucose, 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B and $^{45}\text{Ca}^{2+}$ for various periods of time. The rate of $^{45}\text{Ca}^{2+}$ uptake increased linearly with time for about 120 minutes after which it became stationary. There was a significant stimulation in the uptake of $^{45}\text{Ca}^{2+}$ after the addition of A. bisporus PHA-B in the medium (17.5 μg/ml) and thus at this lectin concentration the stimulation of $^{45}\text{Ca}^{2+}$ uptake is more than 12.5 folds as compared to the control (2 mM glucose).

It may be concluded that $\text{Ca}^{2+}$ plays a vital role in stimulus secretion coupling and the insulin release and $^{45}\text{Ca}^{2+}$ uptake is affected by the lectin (A. bisporus PHA-B). If insulin release
is controlled by ionic events in or near the B-cell membrane. The action of the lectin would be to bind directly to the plasma membrane or the traditional receptors. The stimulation of 45Ca2+ uptake by lectin also suggests that the lectin induces alterations in the structure and arrangement of specific membrane component may in some way facilitate the process of 45Ca2+ uptake and the process of exocytosis of hormone release.

**Insulin Release in Aging in Response to Agaricus Bisporus PHA-B from Islets of Langerhans**

Maturity onset diabetes mellitus may be caused due to altered insulin secretion and biosynthesis, increased adiposity and altered insulin action. It has been demonstrated that glucose mediated insulin release is diminished in aging.

For in vitro studies of the effect of A. bisporus PHA-B on insulin release and 45Ca2+ uptake by the islets isolated from 1, 3, 6 and 12 month old rats were incubated with 2 mM glucose, 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B and 45Ca2+. It was found that the basal insulin release in the presence of 2 mM glucose was almost the same in all the group of islets irrespective of the age of the rats.

The A. bisporus PHA-B stimulated insulin release quite significantly in younger animals. The increase in the release of insulin in the presence of lectin in the case of islets
isolated from one month old rat was 6 folds as compared to the control (2 mM glucose alone) whereas the increase in the insulin release in the presence of the lectin was 2.4 folds as compared to the control in the case of islets isolated from 12 month old rat. There was also a 1.5 fold increase in insulin release in the case of islets of 1 month old rat as compared to the islets of 12 month old rat in response to lectin (A. bisporus PHA-B).

It may be concluded that insulin release in aging in response to A. bisporus PHA-B follows the same pattern as the glucose stimulated insulin release and if the concept of specific interaction of A. bisporus PHA-B and receptors leading to conformational changes in the structure of the membrane of B-cells is accepted, then it would be conceivable that aging has some effect on the structural or conformational integrity of B-cell membrane which would lead to alteration in the responsiveness of B-cell to A. bisporus PHA-B.

$^{45}\text{Ca}^{2+}$ UPTAKE BY ISLETS OF DIFFERENT AGE GROUPS IN PRESENCE OF AGARICUS BISPORUS PHA-B

The corresponding $^{45}\text{Ca}^{2+}$ uptake studies were carried out by islets isolated from 1, 3, 6 and 12 month old rats in response to 2 mM glucose and A. bisporus PHA-B. It was found that like insulin secretion the $^{45}\text{Ca}^{2+}$ uptake by islets in response to 2 mM glucose did not alter with age and when the incubation
period was increased from 1 hour to 2 hour there was no change observed in the uptake.

The stimulation of $^{45}$Ca$^{2+}$ uptake by islets in response to A. bisporus PH4-B (17.5 /µg/ml) was maximum in the islets isolated from younger animals as compared to the older ones. The uptake higher in response to lectin was 54.5 fold in the case of islets isolated from 1 month old rat as compared to the control (2 mM glucose) whereas the increase in $^{45}$Ca$^{2+}$ uptake was 20.4 fold in the case of islets of 12 month old rat as compared to the control. There was an increase in lectin stimulation of $^{45}$Ca$^{2+}$ uptake in the case of islets of 1 month old rat by 3 fold as compared to the islets which were isolated from 12 month old rat.

The conclusion may be drawn that $^{45}$Ca$^{2+}$ uptake is probably a prerequisite for the stimulatory action of A. bisporus PH4-B for insulin secretion and the interaction of PH4-B and its receptor which leads to conformational changes in the B-cell membrane would affect $^{45}$Ca$^{2+}$ uptake and consequently exocytosis of insulin. However, more work is required to understand the exact mechanism of A. bisporus PH4-B mediated insulin release.

**EFFECT OF STREPTOZOTOCIN-NICOTINAMIDE INTERACTION ON PROINSULIN BIOSYNTHESIS IN ISOLATED ISLETS OF LANGERHANS: EFFECT OF LECTINS THEREON**

Streptozotocin is a known diabetogenic agent whose toxic
action can be reversed by nicotinamide. In order to study the
effect of streptozotocin-nicotinamide interaction these studies
were performed by incorporation of $^{14}$C-leucine into acid-ethanol
extractable proteins (proinsulin/insulin).

It was found that by increasing the concentration of
streptozotocin from 0.05 to 1.5 mg per 60-70 islets, the bio-
synthesis of total islet protein decreased correspondingly as
compared to the control (non-streptozotocin exposed islets)
and at streptozotocin concentration of 1.5 mg per 60-70 islets
the total protein biosynthesis was retarded by 5.5 fold as
compared to the control. By increasing the concentration of
nicotinamide from 0.5-4 mg per 60-70 islets, keeping strepto-
zotocin concentration constant (1.5 mg), the inhibitory action
of streptozotocin was reversed and at nicotinamide concentra-
tion of 4 mg in the medium the protein biosynthesis was restored
to control value.

Similarly, the addition of streptozotocin (0.05-1.5 mg/
60-70 islets) in the medium resulted in the inhibition of $^{14}$C-
leucine incorporation into acid-ethanol extractable proteins
(proinsulin). It was found that at streptozotocin concentra-
tion 1.5 mg/60-70 islets, there was 79% inhibition of the
incorporation of the tracer into proinsulin and thus the proinsulin
biosynthesis was retarded to 21% as compared to the control.

For protective studies nicotinamide (0.5-4 mg/60-70 islets)
was incubated at constant concentration of streptozotocin
It was observed that at nicotinamide concentration 4 mg/60-70 islets, 100% protection was achieved against the streptozotocin toxicity.

There was no significant change in the incorporation of $^{14}$C-leucine into islet proteins and proinsulin in the presence of *A. bisporus* PHA-B. There was a 11% decrease in the incorporation radioactivity in acid ethanol soluble protein as compared to the control. The decreased radioactivity counts in the acid-ethanol extracts of islets may be due to the release of stored hormone in the B-cells.

It may be concluded that streptozotocin a diabetogenic agent, inhibits the proinsulin biosynthesis by depressing NAD$^+$ which is required by poly (ADP-ribose) polymerase for the repair of DNA, the damage caused by streptozotocin. Nicotinamide a precursor of NAD$^+$ may be preventing the decrease of NAD$^+$, thus maintaining the level of poly (ADP-ribose) polymerase which protects DNA from damage thus protecting proinsulin biosynthesis. Alkylation of DNA bases in the pancreas by streptozotocin may be another explanation for the inhibition of proinsulin biosynthesis, whereas nicotinamide reduces the extent of methylation and thus preventing proinsulin biosynthesis. *A. bisporus* PHA-B does not exert any effect on the toxic action of streptozotocin, probably due to their different sites of interaction on B-cell membrane.
Cholecystokininins are located in central and peripheral nerves and their nerve terminals have been found in pancreatic islets which have known insulinotropic property. Among CCKs, CCK-4 (Try-Met-Asp-Phe-NH$_2$) is the most effective one. CCK-4 and its two analogues Glp-Met-Asp-Phe-NH$_2$ and Pro-Met-Asp-Phe-NH$_2$ were synthesized in this laboratory and were studied with respect to insulin releasing property.

The insulin release from the islets of Langerhans at the CCK-4 (Trp-Met-Asp-Phe-NH$_2$) concentration of $10^{-10}$M was $68 \pm 8 \mu$U/5 islets/hr as compared to the control (2 mM glucose) which was $30 \pm 5 \mu$U/5 islets/hr. The insulin release increased linearly with the concentration of the tetrapeptide. When tryptophan of the CCK-4 was replaced by $p$-glutamate the tetrapeptide formed (Glp-Met-Asp-Phe-NH$_2$) was not that potent. This tetrapeptide was active at higher concentration of $10^{-8}$M.

Similarly when tryptophan of the CCK-4 was replaced by proline the tetrapeptide thus formed (Pro-Met-Asp-Phe-NH$_2$) also lost the activity. It was active at concentration not less than $10^{-8}$M.
It may be suggested that tryptophan is necessary for the expression of insulin releasing activity of the CCK-4. Any substitution on N-terminal may be the cause of less activity as shown by the present studies. However, it will be of considerable interest to test the insulinotropic activity of these analogues on other animal species as CCKs are species specific for their activity.
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To my lovable and kind-hearted parents whose love, affection, patience and devotion made my efforts succeed.
This is to certify that the work embodied in this thesis has been carried out by Mr. Nafees Ahmad under my supervision. He has fulfilled all the requirements for the degree of Doctor of Philosophy in Biochemistry of the Aligarh Muslim University, Aligarh, regarding the nature and period of investigational work. The work included in this thesis has not been submitted for any other degree, and unless otherwise stated is all original.

(J.R. Kidwai)
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November 20, 1982. 

Nafees Ahmad
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PREFACE

During the sixty years following the discovery of insulin by Banting and Best steady progress has been made in elucidating the physiologic action and the chemical nature of the hormone, but it was only during the last two decades that research has been centred around the problems of biosynthesis, storage and release of insulin. It appears that regulatory system for insulin secretion is subserved by multiple mechanisms and is highly complex. Consequently, investigators should anticipate the possibilities of a variety of regulatory sequences controlling the secretion of insulin in response to different secretagogues. Of utmost significance in this context is the nature of the receptors on the B-cell membrane which make possible for some extracellular factors to influence the intracellular events in the B-cell.

The work which form the subject matter of this dissertation is the outcome of authors efforts in the direction of obtaining some information about the effect of aging on the
B-cell membrane of rat, the effect of streptozotocin toxicity on proinsulin/insulin biosynthesis and its reversal by nicotinamide and the effect of gastrointestinal hormone cholecystokinin on insulin release from B-cells. The problem was approached by employing a lectin purified from white mushroom Agaricus bisporus which binds on B-cell resulting in the stimulation of insulin release.

The results reveal some new findings on lectin potentiated insulin release and calcium uptake and the effect of age on the susceptibility of B-cells to potentiators with respect to insulin release and calcium uptake.

Many questions remain to be answered. If and when the missing pieces of the puzzle are to be found, it may be possible to determine the etiology of diabetes and with such knowledge, eventually its cure.
ABBREVIATIONS

<table>
<thead>
<tr>
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<tr>
<td>AMP</td>
<td>Adenosine-5'-'monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-3'-'5'-'diphosphate</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspargine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine-3'-'5'-'monophosphate</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetracetic acid</td>
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<tr>
<td>Glp</td>
<td>p-glutamate</td>
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<tr>
<td>HAU</td>
<td>Hemagglutination unit</td>
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<td>hr</td>
<td>Hour</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>mCi</td>
<td>millicurie</td>
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<td>Met</td>
<td>Methionine</td>
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<tr>
<td>mM</td>
<td>millimoles</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>POPOP</td>
<td>Dimethyl 1,4 bis (2-(5-phenyl oxazole)-benzene</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
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contd...
RNA = Ribonucleic acid
rpm = Revolution per minute
SZ = Streptozotocin
TCA = Trichloroacetic acid
Tris = (Hydroxymethyl)aminoethane
Trp = Tryptophan
μCi = microcurie
μg = microgram
μM = micromoles
μU = microunits

++++++
*************
INTRODUCTION
*************
Diabetes mellitus is a very common metabolic disorder affecting above 2% of our population. It is one of the diseases against which there is, as yet, no effective treatment for permanent cure. Hyperglycemia and glycosurea are the main symptoms of this chronic disease. It is also known as disease of the pancreas.

The relation of diabetes with pancreas was first established by Von Mering and Minkowsky in 1889. This led to the discovery of insulin by Banting and Best in 1922. The discovery of insulin also provided the final evidence that pancreas was the source of its internal secretion and was responsible for the regulation of blood glucose level. Insulin was the first protein to be crystallized (Abel, 1926) and sequenced (Sanger and Thompson, 1953). Again, it was the first protein hormone whose tertiary structure had been elucidated (Adams et al., 1969; Blundell et al., 1971).
Although extensive research work is being carried out with the view to the understanding of the nature and function of the individual endocrine cells, the entire cellular composition and the exact mechanism of insulin release from B-cells (Beta cells) still remains to be fully elucidated.

**THE ENDOCRINE PANCREAS**

The endocrine part of the mammalian pancreas is composed of small clusters of cells - the islets of Langerhans - named by Laguesse in 1894 after its discoverer Paul Langerhans (1869).

It was later established that islets of Langerhans were endocrine glands and their hormonal secretions help carbohydrate metabolism. The islets of Langerhans are irregularly distributed in the pancreatic parenchyma. Their number is quite variable, but has been estimated at about 1,000,000 for the pancreas of most adult human (Ogilvie, 1937). The total volume of the tissue represents 2 to 3% of the whole gland and total weight amounts to 1-2 g in adult human (MacLean and Ogilvie, 1955; Gepts, 1957, 1958). The islets vary considerably in size, averaging 100-200 μ in diameter. Small islets are more numerous but the major part of the endocrine pancreas corresponds to medium-sized islets (Hellman, 1959). Each islet is supplied by one to three arterioles and drained
by one to six renules (Bunnag *et al.*, 1963). The islet cells are separated from the blood stream by the largely fenestrated membranes, one closely applied to epithelial cells, the other underneath the endothelium. In between normal islets show a few pericytes, fibrocytes, histocytes and mast cells (Estermark, 1973).

**CELLULAR COMPOSITION OF THE ENDOCRINE PANCREAS**

Although more than one hundred years have passed since the discovery of pancreatic islets, our knowledge of their cellular composition is still incomplete. Already in 1907, Lane demonstrated the existence of two main types of islet cells, the A - (Alpha) and B - (Beta) cells. A third type, the D-cell was discovered by Bloom in 1931. In the pancreas of normal adult humans, the B-cells contribute for about 75% of the total islet cell population whereas A- and D-cells represent 20% and 5% respectively. However, many pathological conditions besides diabetes, many cause significant variation in these figures. The response of A- and B-cells to different stains have led to considerable confusion about the nomenclature of the islet cells. The specificity of some of the stains of A-cells have been questioned. It has been shown that certain techniques not only stain all non-B-cells but a variable amounts of B-cells as well (Creutzfeldt, 1953; Gepts, 1957). On the other hand, with certain techniques less A-cells
are stained (Hellerstrom and Hellman, 1960). \(A\)-cells have been subclassified into \(A_1\)- and \(A_2\)-cells. \(A_1\)-cells are those that take up silver and \(A_2\)-cells remain unstained. \(A_2\)-cells are stained by using silver impregnation technique as recommended by Grimelius (1968) but it also stains few \(A_1\)-cells. The relationship of \(A_1\)-cells to the D-cells of Bloom is still controversial. Most authors claim that \(A_1\)-cells and D-cells are identical (Fiyita, 1964; Apple, 1968 and Grieder et al., 1970), but divergent opinions have been expressed. Van Assche (1970) has pointed out that not all D-cells are stained by the Hellman - Hellerstrom silver technique and has expressed the opinion that the D-cells of Bloom constitute a heterogeneous group of islet cells, some of which still await identification. Like (1967) even refused to recognize a third cell type, and regarded the D-cell as a viable but altered \(A\)-cell. Grieder et al (1970) firmly opposed this view and presented the D-cell as a distinct and functionally independent cell type. Deconinck et al. (1971, 1972) in agreement with the findings of Van Assche (1970), expressed the opinion that besides \(A\)- and \(B\)-cells, the islets contain two other cell types which refer to as type III and type IV cells. The four types of cells were called respectively \(A\), \(B\), \(D\) (corresponding to the type III cells of Deconinck et al., 1971, 1972) and \(D_1\) (equivalent to the type IV cells of the same authors). It was pointed out that cells equivalent to \(D\)- and \(D_1\)-cells are normally present in the
gastroduodenal mucosa (Solicea et al., 1973). It was also reported that cells identical to pancreatic A-cells also occur in the same mucosa, at least in some species (Unger and Orci, 1975; Dobbs et al., 1975; Larsson et al., 1975). B-cells on the other hand have never been detected in the mucosa of mammals.

FUNCTIONS OF THE ISLET CELLS

Specific functions of different groups of cells of the islets of Langerhans are only partly understood. It has been accepted since long time that B-cells are responsible for insulin formation and release. Among A-cells only A_2-cells secrete glucagon (Hellerstrom and Hellman, 1962; Petersson and Hellman, 1963; Lundquist et al., 1970). The role of A_1-cells is still not clear. Grieder and McGangan (1971) and Lomsky and his colleagues (1969) are of the view that A_1-cells are responsible for the secretion of gastrin, an inhibitor of insulin release. However, Creutzfeldt et al. (1971), Lotstra et al. (1974), Pearse (1975 and Hokfelt et al (1975) have failed to identify gastrin cells in the islets of normal human.

Somatostatin, a hormonal tetra decapeptide isolated from hypothalamus inhibit release of growth hormone from the anterior lobe of the pituitary has been found to be released by D- (or type III) -cells (Brazeau et al., 1973). It has also been called somatotrophin release inhibiting factor or SRIF.
Systematic studies with the aid of an antibody raised in rabbit against SRIF have revealed that this hormone also exists in several peripheral tissues, including the pancreatic islets (Hokfelt, 1973; Dubois, 1974; Luft et al., 1974; Arimura et al., 1975; Dubois et al., 1975). The discovery of somatostatin (or somatostatin like) cells in the peripheral organs has arisen considerable interest, because this hormone has been shown to inhibit many other secretory process apart from growth hormone release such as secretion of insulin and glucagon (Alberti et al., 1973; Chen et al., 1974; Curry et al., 1974; DeVane et al., 1974; Efendic et al., 1974; Fujimoto et al., 1974; Gerich et al., 1974a,b; Koerker et al., 1974; Mortimer et al., 1974; Sakurai and Unger, 1974). The post inhibitory overshoot of insulin release after somatostatin may be produced in vitro by suppressed action of stimulatory hormone such as gastric inhibitory polypeptide (GIP) and prior infusion with GIP can also potentiate glucose stimulated insulin release (Johnson and Conley, 1980). The close topographical relationship of somatostatin containing cells with B- and A-cell evokes the possibility of a local regulation of the release of insulin and of glucagon by these cells.

The function of D-Type (or type IV) cells is still not clear. A possible candidate for secretion of this cell type is the "pancreatic polypeptide" first isolated by Kimmel et al. (1968, 1971), as a contaminant of chicken insulin. Peptide
similar to the avian pancreatic polypeptide (APP) have been isolated from bovine, ovine, porcine and human pancreas (Langsley et al., 1973). Only a few of the physiological properties of this newly isolated pancreatic polypeptide are known such as stimulation of gastric secretion and of liver glycogenolysis, depression of lipolysis (Lin and Chance, 1972; Hazelwood et al., 1973).

The effect of spontaneous diabetes on pancreatic A-, B- and D-cells were assessed at various stages of diabetic syndrome. Tannbaum et al. (1981) found that the loss of B-cells were evident at all stages of diabetes. They also suggested that A- and D-cells may be affected by the same process which destroyed B-cells. McEvoy (1981) quantitated the changes in the volume of A-, B- and D-cells population in the pancreas islet during postnatal development. The volumes of A- and D-cells reached their maximum values by day 50 and 35 respectively whereas the volume of B-cells and insulin content were highest on day 210 irrespective of sex (McEvoy, 1981). It was concluded that the volume of B-cell may continue to increase with age while A- and D-cells apparently do not. Thus, the considerable confusion which has existed until recently about the cellular composition of pancreatic islets is progressively disappearing.
The islet cells are arranged as cords along the capillary channels within the islets. Individual cells are surrounded by distinct plasma membranes. By freeze etch electron microscopy, intramembranous particles have been demonstrated in the plasma membrane of islet cells. The number of these particles decreases in the B-cells of severely diabetic Chinese hamsters (Orci et al., 1974). The chemical composition and possible role in hormone action, of these particles, is not known yet. The plasma membranes of adjacent islets are attached at special focal points by desmosomes. Gap junction has been demonstrated between A- and B-cells as well as individual B-cells. The function of these gap junction is not clear at present.

The secretory granules of islet cells contain stored hormone products surrounded by smooth membranous sacs (Fig. 1). The mitochondria of islet cells are relatively small in comparison of mitochondria of adjacent acinar cells. Islet cells contain varying amount of rough endoplasmic reticulum (RER) and numerous polysomes scattered throughout the cytoplasm. The Golgi complex is generally located near the nucleus and consists of parallel arrays of smooth membranes and numerous small vesicles. The size of the Golgi complex varies with the functional status of the individual cells (Lacy and Greider, 1979). Insulin immunoreactive sites have been localized in the Golgi apparatus of pancreatic B-cells by light and electron
Fig. 1 - Electron micrograph of a beta cell in a monolayer cell culture of neonatal (2.5 days) rat pancreas. Rows of microtubules (arrows) separate columns of beta granules. Microfilaments (mf) are also present. x 35,000 (Orci et al., 1973).
microscopy (Rarozzola et al., 1981). The demonstration of insulin immunoreactivity in the Golgi apparatus provides direct evidence for the involvement of this compartment in the transport and maturation of proinsulin to insulin.

In normal islet cell, there are relatively few lysosomes. Numerous ceroid bodies are present in the islet cells of the normal adult human pancreas. These intracytoplasmic bodies are autofluorescent and contain lipid and lysosomal enzymes. The origin of these structures is unknown (Lacy and Greider, 1979).

A microtubule-microfilament system is present in the islet cells. The cytoplasmic processes, containing long bundles of microtubules which separate linear columns of β-granules have been shown in B-cells in monolayer cultures (Fig. 2) (Orci, 1974). Microfilaments (50-90 A° in diameter) are present as a web just beneath the plasma membrane and probably occur as individual fibres in the interior of the cell (Fig. 2) (Orci, 1974). In monolayer culture of islet cells, action has been demonstrated beneath the plasma membrane of these cells by immunofluorescent technique (Greider et al., 1974).

**FUNCTIONAL MORPHOLOGY OF B-CELLS**

The demonstration of an impaired insulin secretion in diabetes (Yalow and Berson, 1960; Cerasi and Luft, 1963, 1967) has focused considerable attention on the regulatory mechanisms
Fig. 2 - Electron micrograph of normal beta cells. (A) The cell web of microfilaments is present just beneath the plasma membrane and extends into microvillous processes (mv). At higher magnification, the network of microfilaments of the cell is demonstrated more clearly. Microvesicles (mv) and ribosomes (r) are present in the cell with secretory granules x 125,000 (Orci et al., 1972).
in the function of the B-cells and on the intracellular aspects of the insulin secretory process with the hope of localizing the primary defect in diabetes. The various morphological aspect which appear to be involved in the B-cell response to glucose have been described in several excellent review articles (Steiner et al., 1972, 1974; Randle and Hales, 1974; Lacy, 1975; Hedestock, 1980; Taljedal, 1981). A distinction has been made between the receptor unit, recognizing the B-cell regulators, and the effector unit responsible for the cell response.

The receptor unit

Although the development of radioimmunoassay (Yalow and Berson, 1960) and the techniques for the isolation of viable islets of Langerhans (Hellerstrom, 1964; Lacy and Kostinovsky, 1967; Feldman and Chapman, 1975; Horaguchi and Merrell, 1981) have helped considerably in the understanding of various events in B-cells in the process of insulin release, the exact mechanism through which extracellular factors influence the intracellular events of the endocrine pancreas still remain poorly defined. Recently, methods have been developed for the separation of A-, B- and D-cells from isolated islets of Langerhans (Pipeleers et al., 1979, 1981). This will facilitate the studies on the receptors and also help in understanding in more details, the mechanism of insulin release from the B-cells. In view of the receptor studies in other tissues, it is
generally believed that hormones and neurotransmitters modulate islet function via interactions with membrane receptors. Such hypothetical model has been experimentally documented by Ewart et al. (1975), who demonstrated a parallelism between the binding of mushroom lectins to islets and the associated insulin and glucagon release. Further studies are, however, required on the existence and possible activation of islet membrane receptors. The desire to determine both the nature and localization of the receptor unit in B-cells has initiated the search for enzyme activities, in the islet section, homogenates, and subcellular fractions. In addition to the enzymes involved in various metabolic pathways (Matschnisky, 1962; Randle and Hales, 1972), islet homogenates contain both adenyl cyclase (Atkins and Matty, 1971; Davis and Lazarus, 1972; Kuo et al., 1974) and guanylyl cyclase (Howell et al., 1974) system, which have been located on plasma membranes (Howell and Whitfield, 1972). The message of extracellular factors might thus be transmitted through the intracellular messengers cyclic AMP or cyclic GMP, which modulate insulin synthesis and/or release vis-à-vis, as yet, an undefined mechanism. It has been postulated that glucose interacts also with a membrane receptor related to adenyl cyclase system (Cerasi and Luft, 1970; Matschinsky and Ellerman, 1973) and that a defect in this signal transmission might represent the central defect in diabetes (Cerasi and Luft, 1970). Although glucose does indeed stimulate cAMP
formation in islets of Langerhans (Charles et al., 1973; Grill and Cerasi, 1974) the effect of glucose upon islet function cannot be explained as obtained by cAMP alone (Malaisse, 1973; Hellman et al., 1974). The demonstration that d-D glucose is more insulinotropic than β-form (Niki et al., 1974; Grodsky et al., 1974) is not associated with facilitated glucose transport and metabolism (Taljedal, 1975) but is accompanied with increased cAMP formation Grill and Cerasi (1975) stresses, however, the view that at least a part of glucose effect is carried out throughout cAMP formation.

The effector unit

Both A- and B-cells are equipped with a secretory device capable of producing a fast as well as sustained hormone release (Grodsky et al., 1967; Iverson, 1971). The synthetic machinery of B-cell can also be quickly turned in (Steiner et al., 1972), such immediate cell responses do not only require a fast transmission of the stimulatory signal, but also the existence of some synthetic and secretory effector units in which the various organelles can be rapidly activated.

Proinsulin biosynthesis

It is now generally recognized that most small polypeptide hormones are derived via cleavage of larger precursor molecules (Fig. 3). These larger forms may fulfil a number of important
Fig. 3 - Simplified scheme of the molecular biology of insulin formation. The proinsulin gene is represented schematically in the upper panel. RNA polymerase is necessary for the transcription of proinsulin m-RNA from the gene and this then serves to guide the formation of proinsulin chains on the polyribosomes via the transient proinsulin (dashed lines indicate presequence) (Steiner, 1976).
functions in biosynthesis and secretion (Steiner, 1976). It is now well established that proinsulin, in common to many other exportable proteins, is synthesized by ribosomes associated with rough endoplasmic reticulum (Permutt and Kipnis, 1972) and comprises the major biosynthetic product found in microsomal fraction (Sorenson et al., 1970). However, in vitro translation of mRNA from islets yields a product larger than proinsulin (Lenmark et al., 1976; Lomedeco and Saunders, 1976; Chan et al., 1976). This larger product preproinsulin consists of proinsulin with an amino terminal extension of 20-25 residues with hydrophobic side chains. In proinsulin, such a region most likely would include the $\alpha$-helical region near the N-terminus of B-chain (Blundell et al., 1972). These clustered hydrophobic residues of preproteins may interact with lipid rich membranes to induce the formation of ribosomes-membrane junctions and thereby to facilitate the vectorial discharge and sequestration of the secretory product (Blobel and Dobberstein, 1975).

The molecular weight of preproinsulin mRNA is approximately 210,000 corresponding to a length of about 600 nucleotides (Dugid et al., 1976). Since only 330 nucleotides are required to code for preproinsulin, this mRNA, like most eukaryotic mRNAs, must contain additional nucleotide sequence, which is not translated in addition to a 3'-polyadenylic acid tail. These regions are of particular interest since they undoubtedly include
sequences which regulate the translation of messenger molecule. The nucleotide sequence analysis has revealed a sequence of 13 amino acids immediately preceding the N-terminus of proinsulin and has confirmed the existence of non-coding 3'-terminal segment of 57 nucleotide preceding the 3'-poly A tail (Ulbrich et al., 1977). All the known mammalian proinsulins (Fig. 4) have pairs of basic residues at either end of the connecting polypeptide chain (Arg-Arg between C-peptide and B-chain, Lys-Arg between C-peptide and A-chain) which links the connecting polypeptide to the insulin chain. These residues are excised during the conversion of proinsulin to insulin.

HORMONE TRANSPORT

From endoplasmic reticulum to Golgi complex

Similar to exocrine pancreatic cells both A- and B-cells channel their newly synthesized secretary proteins through RER to Golgi apparatus (Howell et al., 1969, 1974). This energy requiring step (Howell, 1972) has been described to occur along smooth surfaced endoplasmic reticulum budding off into microvesicles, which convey the secretory products to Golgi complex (Orci et al., 1973). After 10-30 minutes, the newly formed proinsulin reaches the Golgi tubules, which have been implicated in the formation of new secretory granules (Munger, 1958).
Fig. 4 - Structure of bovine proinsulin showing sites of cleavage by trypsin (Kimmel et al., 1972).
From Golgi complex to plasma membrane

Secretory vesicles emerge from the Golgi apparatus, where the newly synthesized products are packaged into the granule case and surrounded by Golgi membranes. It is nevertheless believed that the newly formed granules undergo a maturing process over the next 45-150 minutes (Howell et al., 1969a; Orci et al., 1973) during which the conversion process will continue within the secretory vesicles (Kemmler et al., 1972). No preferential release of newly formed proinsulin has been detected in the rat isolated islets (Sando et al., 1972; Sando and Grodsky, 1973) which is consistent with the concept that all secretory proteins are transported and released via a similar mechanism.

Conversion of proinsulin to insulin

In the process of conversion of proinsulin to insulin, the possibility that the converting enzymes are related to some of the lysosomal proteases has been considered by several workers (Steiner et al., 1972; Smith, 1972 and Puri et al., 1978). Various carboxypeptidases active in acid pH range have indeed been reported including catheptic carboxypeptidase B (Greenbaum and Yamafugi, 1965), possibly associated with cathepsin-B_2 (Ninjoor et al., 1974) and bradykinase (Greenbaum and Yamafugi, 1965). When insulin is liberated from proinsulin, it evidently tends to crystallize with zinc (Steiner, 1973). It is possible that these metal ions might influence the
conversion process in some way and then subsequently help in the sequestration of the newly formed insulin in an osmotically inactive and biochemically stable crystalline form. The cationic arginine and lysine residues liberated during conversion of pro-insulin may diffuse from the granules to be replaced by hydrogen and zinc ions. The C-peptide liberated in the conversion process probably remains in the clear space (Rubenstein et al., 1969; Steiner et al., 1971) surrounding the dense insulin crystal.

Recently in our laboratory it has been shown that total in vitro conversion of proinsulin to insulin in the islets can be achieved by cathepsin B (Puri et al., 1978) and this conversion is inhibited by cathepsin B antibodies (Bansal et al., 1980). Histochemical evidence for the presence of cathepsin B like enzymes in the B-cell of pancreas has been provided by Smith et al. (1972). Kemmler et al. (1973) have shown that proinsulin is converted to insulin in the secretory granule fraction prepared from rat islet of langerhans.

The important products of conversion, insulin and C-peptide are stored in β-granules till they are secreted as shown by immunohistochemical studies (Misugi et al., 1970).

Little information is so far available regarding the fate of secretory vesicles, after they left Golgi complex and before they fuse with the plasma membranes. It is generally conceived that newly formed granules are pinched off from the Golgi tubules
and mix with a pool of pre-existing storage granules, out of which some migrate to the plasma membrane in a random fashion (Sando and Steiner, 1973)

Emiocytosis

The emiocytosis process has been originally described by Palade in the pancreatic acinar cells, which appeared to discharge their secretory proteins following fusion of the zymogen granule membrane with the plasma membrane (Palade, 1958). Lacy extended this phenomenon to the B-cells in which frequent occurrence of emiocytosis in tandem was emphasized (Lacy, 1961). It also appeared that calcium was a universal prerequisite for this release mechanism (Rubin, 1970).

ROLE OF MICROTUBULE-MICROFILAMENTS IN THE TRANSPORT OF INSULIN

A microtubular microfilamentous system is present in B-cells. The microtubules are scattered throughout the cytoplasm. Lacy et al. (1966, 1972) and Malaissee et al. (1971a) demonstrated that colchicine, vinblastine and vincristine (agents which destroy microtubules) inhibited the release of insulin following stimulation of amino acid transport in isolated rat hepatocytes (Prenlki, 1981). It was proposed that microtubular microfilamentous system was involved in B-cell secretion and that
the microtubules were responsible, in some way, for the translocation of the \( \beta \) -granules to the cell membranes. This concept could explain how a column of cell granules could be released by emiocytosis at a single locus on the plasma membrane of B-cells. This could also explain the biphasic pattern of insulin release since these granules associated with the microtubular system would be released initially in the first phase, whereas additional stored granules, as well as newly formed \( \beta \) -granules would become associated with the microtubular system and form the second phase (Lacy, 1970).

The idea of the participation of the contractile elements in the secretory mechanism has found support by the similarity between calcium movement during secretory and contractile process. The existence of actin and myosin in secretory cells further elaborates this hypothesis (Abramowitz et al., 1972; Blitz and Fini, 1974). The final step in the release of \( \beta \) -granules and therefore of insulin from B-cells is by simple process of emiocytosis (Lacy, 1970a). Based on ultrastructural studies and subcellular fractionation of islet cells, the \( \beta \) -granules enclosed in their smooth membranous sacs, move to plasma membrane, where the membrane enclosing the granule fuse with the plasma membrane. The rupture of the plasma membrane at the point of fusion, results in the liberation of granules to the extracellular space where they get dissolved (Fig. 5) (Steiner, 1976). Using freeze-etch electron microscopy,
BETA GRANULE FORMATION

Fig. 5 - Beta granule formations. The time scale on the right hand side of the figure indicates the time required for each major stages of biosynthesis. (Steiner, 1976).
Orci et al. (1972) have demonstrated the actual fusion of the membranous sac enclosing the β-granules with the plasma membrane. This has also been referred to as exocytosis by many investigators. The mechanism of fusion of the membranous sac enclosing the granules with the plasma membrane is, however, not clear.

**AGENTS INFLUENCING INSULIN RELEASE**

The endocrine cells have a unique property of being able to respond to a specific physiological stimulus with a resultant release of stored hormone in a modulated fashion. The mechanism through which extracellular factors govern the intracellular events in the endocrine pancreas remain poorly defined. The secretion of insulin by pancreatic B-cells is stimulated by nutrients (Sugars, amino acids, fatty acids and ketone bodies), hormones (glucagon and gastric inhibitory peptide), cAMP and its dibutryl derivative, methyl xanthises and by the inhibitors of cAMP phosphodiesterase. Some of these compounds are fully competent to elicit release of insulin and are termed as initiators. These include glucose, mannose, glycer aldehyde and some amino acids e.g. leucine. Other secretagogues amplify the response to initiators are ineffective in the absence of an initiator and are termed as potentiators. These include the hormones, cAMP, methyl xanthises, some sugars (fructose, N-acetyl glucosamine and some amino acid L-arginine, L-phenyl alanine.
There are also some insulin secretagogues which require an initiator (glucose) for insulin release. Some potentiators can be induced to initiate release under special conditions such as theophylline and dibutryl cAMP.

GLUCOSE

The stored insulin in normal human being is approximately 200 units. When the concentration of glucose is increased above a level of 100 mg%, the B-cell respond by releasing the insulin in a triphasic manner. The rate of insulin release increases immediately after raising glucose level above critical point and reaches a maximum 4-5 minutes after the stimulation. The rate of release declines, achieves a second maximal level approximately 10-15 minutes after stimulation and this rate is maintained until the glucose concentration is returned to normal levels (Cerasi et al., 1974). The maximal levels of secretion of these two phases is achieved by glucose concentration of 500 mg%. High glucose concentrations do not produce any further increase in the rate of insulin release. The relationship between extracellular glucose concentration and the rate of insulin release is sigmoidal. Glucose concentrations below approximately 3 mM do not influence the secretory rate. The threshold concentration is around 4 mM. The largest increase in insulin secretion occurs between 4 and 7 mM of glucose, which
means that B-cells are actually sensitive to small changes in glucose concentration within the physiological range (Ashcroft et al., 1972).

When glucose reaches the B-cell three events are generated almost simultaneously although their expression (especially for maximal efficiency to be reached) necessitate different time lapses (Cerasi, 1975). Two distinct recognition sites for glucose give rise to the two positive inputs in the B-cells, initiation of the signal for the firing off of insulin (rapid) and potentiation, that results in amplification of the former effect of the sugar.

The third event, negative feed back regulation generated by insulin itself or some process linked to its release, modulate the final response of the pancreatic islet (Fig 6). The question as to whether glucose acts via a metabolite or a direct receptor is unresolved.

The term glucoreceptor is used to describe the molecular species in B-cell, that, by virtue of its ability to bind glucose, confers on the B-cells sensitivity to changes in the extracellular glucose concentration. Two models have been proposed for B-cell glucoreceptor (Randle et al., 1968). In regulator site model glucose is envisaged as an allosteric modifier of a plasma membrane bound glucoreceptor, presumably of protein nature, that is able to bind glucose and other
Fig. 6 - Hypothetical model for B-cell recognition of glucose as an initiator and as a potentiator (Cerasi, 1975).
initiating sugars. Combination of sugar with receptor leads to alterations in the B-cell that, presumably via changes in membrane permeability, culminate in the influx of Ca\textsuperscript{2+} and triggering of exocytosis. In the substrate site model the metabolism of glucose and other sugars within the B-cell presumably provides one or more intracellular signals (glycolytic intermediates, or cofactors) that initiate an increase in the cytoplasmic concentration of free Ca\textsuperscript{2+} and thereby exocytosis. In this model the glucoreceptor is an enzyme or a system of enzymes that catalyzes the rate limiting step in the B-cell metabolism of glucose to these intracellular signals. These two models are not considered to be mutually exclusive. Different combinations of the two systems are conceivable. Formulation of these models greatly stimulated the study of B-cell function and metabolism but also created considerable controversy during recent years.

**ADENYL CYCLASE**

Agents which are known to increase cAMP content in B-cells also enhance the secretion of insulin (Malaisse et al., 1967; Lambert et al., 1971). Similarly elevation of cAMP level has been demonstrated in isolated islets stimulated with glucose preferentially by α-anomers of D-glucose (Charles et al., 1973; Grill and Cerasi, 1974, 1975). However, elevation of cAMP levels in the beta cells in conjunction with stimulating
concentration of glucose will result in the enhancement of glucose induced insulin release. The mechanism of potentiation of insulin release by glucose and cAMP is unknown. The cAMP may indeed be mediating the glucose action to the site of insulin release (Charles et al., 1979). It has been recently suggested that glucose potentiates 3-isobutyl-1-methyl xanthine response rather than the reverse (Kranz et al., 1980). Glucose induced insulin release is mediated by increased Ca\(^{2+}\) uptake, decreased Ca\(^{2+}\) efflux and mobilization of stored Ca\(^{2+}\). Glucose may potentiate cAMP response by any or all of these mechanisms (Kranz et al., 1981).

**CALCIUM IONS**

It is now generally agreed that a rise in extracellular Ca\(^{2+}\) of the B-cell is a prerequisite for glucose to exert its stimulating effect on insulin release. Stimulation of insulin secretion, not only by glucose but a variety of other secretagogues, depends on the presence of extracellular calcium (Grodsky and Bennett, 1966; Milner and Hales, 1967; Curry et al., 1968). One exception to this rule is that certain thiol reagents do stimulate insulin release in Ca\(^{2+}\)-free media, but their releasing action is attenuated. Curry et al. (1968) and Hellman et al. (1974) have shown, using the isolated perfused pancreas, that the total amount of insulin release in response to 2 min glucose stimulus was linearly related to the calcium
concentration between 0.25 and 2.5 mM and reached a plateau at about 5 mM. Replacement of extracellular Ca\(^{2+}\) by equimolar of Sr\(^{2+}\) elicited normal glucose stimulation. Beryllium could not replace extracellular Ca\(^{2+}\), 2.5 mM Be\(^{2+}\) inhibited the glucose effect (Hales, 1970). However, when equimolar Ba\(^{2+}\) was used as a substituent cation for Ca\(^{2+}\), insulin release was stimulated (Hales and Milner, 1968). A method that might facilitate the analytical problem was presented by Breemen et al. (1972), who used La\(^{3+}\) to displace extracellular Ca\(^{2+}\) and to block transmembrane fluxes in \(^{45}\)Ca\(^{2+}\) loaded smooth muscle. Chandler and Williams (1979) employed this method in experiments with mouse pancreatic fragments. Hellman et al. (1976) applied this to pancreatic islets in order to study whether glucose induces a net uptake of Ca\(^{2+}\) by B-cells. Thus, glucose stimulated insulin release is Ca\(^{2+}\) dependent, perhaps because Ca\(^{2+}\) couples the process of stimulus recognition to that of insulin discharge (Douglas, 1968; Milner and Hales, 1970; Matthews, 1970; Malaisse, 1973; Malaisse and Pipeleers, 1974). Although several studies have indicated that glucose alters the state of Ca\(^{2+}\) in pancreatic B-cells the nature of changes and the mechanism are poorly understood (Talgedal, 1976). Electrophysiological experiments suggested that glucose increases Ca\(^{2+}\) influx into B-cells (Dean and Matthews, 1970; Meissner and Schmelz, 1974). When mannitol was used as a marker of the extracellular space in the isolated islets, it was observed that glucose enhanced the rate of \(^{45}\)Ca\(^{2+}\)
uptake by islet cells (Hellman et al., 1971).

Malaisse and coworkers (1971, 1972, 1973) did some of the first studies on Ca\(^{2+}\) handling by islets. They initially observed that the retention of radioactivity in rat islets incubated with \(^{45}\text{Ca}^{2+}\) for 90 min and subsequently washed extensively was increased by glucose. Various other stimulatory agents such as mannose, leucine, and sulfonyl urea also caused an accumulation of \(^{45}\text{Ca}^{2+}\). Furthermore, diazoxide, epinephrine and mannheptulose, the inhibitors of insulin release, inhibited glucose stimulated retention of \(^{45}\text{Ca}^{2+}\). Varying concentrations of calcium, verapamil, diazoxide were used for short and long term islet culture (Lainweber and Schatz, 1982) and suggested that the inhibitors of insulin release do not longer influence the release and biosynthesis of insulin independently after a longer culture period in contrast to their short term effects.

A remarkable relationship of proportionality under a great variety of experimental conditions between the rate of glycolysis and net uptake of \(^{45}\text{Ca}^{2+}\) by rat islets was observed and was concluded that glycolysis exerts a tight control on the rate constant for Ca\(^{2+}\) transport across B-cell membrane (Sener et al., 1976). Dibutryl cAMP and theophylline did not modify glucose stimulated Ca\(^{2+}\) accumulation but increased the rate of \(^{45}\text{Ca}^{2+}\) efflux from preloaded islets (Brisson et al., 1972). Thus theophylline provoked a movement of calcium from some intracellular organelle to the cytoplasm. Malaisse et al.
(1973) have postulated that glucose increased the intracellular concentration of $^{45}\text{Ca}^{2+}$ by inhibiting its outward transport. However, it did not preclude the possibility that glucose had caused a fall in the specific activity of the cytoplasmic $^{45}\text{Ca}^{2+}$ alternately, that glucose had increased the influx of $\text{Ca}^{2+}$ into the cells. Hellman et al. (1976), using a technique involving washing of islets with lanthanum solutions in order to displace $\text{Ca}^{2+}$ and to block transmembrane fluxes in $^{45}\text{Ca}^{2+}$ loaded islets from ob/ob mice demonstrated that 20 mM glucose caused a net uptake of $\text{Ca}^{2+}$ from the extracellular fluid. Factors other than an increased $\text{Ca}^{2+}$ uptake may play a crucial role in insulin release. One such factor may be the correct physiological intracellular distribution of $\text{Ca}^{2+}$ after its initial entry through the plasma membrane. Malaisse et al. (1971) have suggested that in glucose induced insulin release, calcium could serve as the intracellular trigger for the induction of contraction or change in physical conformation of the microtubular-microfilamentous system in the $\beta$-cells resulting in the intracellular transport of $\beta$-granules and their release by emiocytosis (Lacy, 1970). Recently, it has been suggested that a new protein kinase system which is coupled with phosphatidylinositol turnover, activated by reversible attachment to membranes in the presence of $\text{Ca}^{2+}$, may be involved in the mechanism of glucose stimulated insulin release (Tawigawa et al., 1981).
AMINO ACIDS

Certain amino acids, viz. L-arginine (Palmer et al., 1975), L-leucine and L-phenylalanine (Landgraft, 1974) have been shown to stimulate the release of insulin by different mechanisms. L-arginine, the most potent amino acid for insulin release stimulates the release indirectly by eliciting glucagon from A-cell (Milner, 1970). Non-metabolizable analogs of leucine and arginine stimulate insulin secretion, which suggest that amino acids may trigger islet hormone secretion via membrane receptor (Efendic et al., 1972). Schafer and Schatz (1981) have shown that leucine in contrast to arginine supports B-cell function, especially insulin biosynthesis during long term culture of islets. Recently it has been shown that the fetal rat pancreas grown in vitro may require both essential and non essential amino acids for the full expression of insulin biosynthesis and release (Milner and DeGaspero, 1981).

FATTY ACIDS

Short chain fatty acids (Octanoate, valerate, butyrate and propionate), long chain fatty acids, (oleate and palmitate) and ketone bodies (β-hydroxy butyrate and acetoacetate) stimulate insulin release from islets of various species in the presence of non-stimulating concentration of glucose (Hawkins et al., 1971). It has been proposed that these agents may be important during starvation to ensure sufficient insulin
release to prevent fetal ketosis (Seyffert and Madison, 1967).

**LECTINS AND INSULIN RELEASE**

Plant lectins are proteins that bind to specific carbohydrate groups on the plasma membranes of mammalian cells, exhibiting a variety of biologic effects. For example, lectins stimulate lymphocytes to undergo blast transformation (Nowell, 1960), induce the release reaction in platelets (Hajeris and Brodie, 1972) and evoke a number of insulin like effects in isolated fat cells (Cutraceasas and Tell, 1973; Czech and Lynn, 1973). Thus the plant lectins were scrutinized with renewed interest and over the intervening years have been found to induce a number of biological responses in variety of cell types, some of which are listed in Table 1. The precise mechanism by which the binding of lectins to plasma membrane produce such alterations in the function of target cell remain unknown but it is clear that these agents can be employed as useful probes for the elucidation of the structural and functional characteristic of mammalian cell. Ewart et al. (1975) have shown that lectins from Agaricus bisporus and Agaricus capestris stimulate insulin and glucagon release in the presence of 2 mM glucose. Agaricus bisporus PHA-B stimulated insulin release was independent of a source of a metabolic energy, but was abolished by duterium oxide. However, the exact mechanism of the A. bisporus PHA-B stimulated insulin release is not known.
<table>
<thead>
<tr>
<th>Biological effect</th>
<th>Phaseolus vulgaris</th>
<th>Lens culinaris</th>
<th>Agaricus bisporus</th>
<th>Concanavalin A</th>
<th>WGA</th>
<th>Ricinus communis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitogenic to lymphocyte</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>Induce platelet release reaction</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Induce insulin release from pancreatic islet cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inhibit phagocytosis by granulocytes</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Inhibit fertilization of ovum by sperm</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Inhibit protein synthesis in mammalian cell</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>
INSULIN RELEASE IN AGING

The rat fetus responds in vivo to a one hour hyperglycemia by increasing its plasma insulin concentration (Girard et al., 1974; Kervran and Girard, 1974; Blazquez et al., 1975; Kervran and Girard, 1976; Kervran et al., 1979). This response is present as early as day 18.5 of gestation (Kervran and Girard, 1974) and exhibits a clear biphasic pattern by days 20.5 and 21.5 (Kervran and Girard, 1976 and Kervran et al., 1979). The biphasic insulin release induced by glucose in vitro is already present in the term rat fetus (Kervran et al., 1977; Randon and Kervran, 1979). It has been reported that islets isolated from rat fetuses at 2 days before term responded to glucose, leucine and arginine during incubation in vitro (Sodoyez-Goffaux et al., 1979). The mechanism controlling biphasic insulin release develops during late fetal life in rat and transition from fetal to adult type of insulin secretion more likely parallels quantitative rather than qualitative, changes within the B-cells (Kervran and Randon, 1980).

The high incidence of hyperglycemia in aged human populations is of significance because of its close relationship to maturity onset diabetes mellitus (Hayner et al., 1965; Boyns et al., 1969; O'Sullivan et al., 1971). Several factors which may play a role in development of this hyperglycemia of aging include, altered insulin biosynthesis and release, increased
adiposity with resulting reduction in insulin sensitivity and altered insulin action. Clinical human studies and in vivo animal studies have revealed decreased, unchanged, or increased glucose stimulated insulin release in aging, and the resulting uncertainty from these studies is still not resolved (Andres et al., 1970; Barbagalls-Sangiorgi et al., 1970; Nolan et al., 1973; Dul and Ensink, 1977; Andres and Tobin, 1977; Davidson, 1979; Yashino et al., 1979; Soerjodibroto et al., 1979). There is a diminished glucose stimulated insulin release from islet of Langerhans of older rats (Coddling et al., 1975; Gold et al., 1976; Kitchara and Adelman, 1979; Reaven et al., 1979), but the cause of diminished insulin secretion has not been determined. Among the possible causes of the aging induced secretory defect are 1) alterations in the enzymes of adenylate cyclase - cAMP system and related protein phosphorylation and 2) decreased activity of enzymes of the islet glycolytic pathway (Lipson et al., 1981).

MEMBRANE RECEPTOR SITES AND INSULIN RELEASE

Previous concept that induction of insulin release was due to the metabolism of glucose has been ruled out by Matschinsky et al. (1971) who have shown that no significant changes in substrate and ATP level could be demonstrated in islets following acute glucose stimulation employing microchemical techniques. They suggested that glucose was inter-
acting with specific receptor on the plasma membrane of B-cells. The problem of establishing the existence of glucose receptor on B-cell membrane is more difficult than for other tissues, since the stimulating agent is not a protein and is transported into the cell. Tomita et al. (1974) using alloxan as a possible probe for the identification of glucoreceptor on the plasma membrane have demonstrated that the exposure of isolated islets to alloxan in vitro for 5 min would inhibit subsequent glucose induced insulin release. Yet tolbutamide induced insulin release remained intact. Concomitant exposure of the isolated islets with alloxan and high concentration of D-glucose (Bansal et al., 1980), D-mannose, or 3-O-methyl-D-glucose resulted in protection of the B-cells against alloxan toxicity (Zawalich and Beidler, 1973; Tomita et al., 1974; Rossini et al., 1975), while L-glucose which is not transported into B-cell showed no such protection. It has also been shown that α-anomer of D-glucose provides protection at a lower concentration than β-anomer (Rossini et al., 1974; McDaniel et al., 1976; Niki et al., 1976). These findings suggest that specific receptor sites for glucose do exist on the B-cell membrane and provide a means to characterize the glucose receptor.

NEURAL INFLUENCES ON INSULIN RELEASE

Insulin release can also be directly influenced by the central nervous system. Both the sympathetic and para-
sympathetic nervous system may be important modulators of islet A- and B-cell function (Wood and Porte, 1974). Nerves to the pancreas contain sympathetic fibres from the greater and middle splandinic nerves, originating in the celiac and superior mesentric plexuses and parasympathetic fibres from the vagus nerve. Adrenergic and cholinergic terminals have been identified by light and electron microscopy at the periphery of islets. Hormonal peptides may also serve important roles as neuroregulators. The related gut hormones, gastrin and cholecystokinin (CCK) belong to this group of peptides. They are located in central (Dockray, 1976; Larsson and Rehfeld, 1977; Muller et al., 1977; Rehfeld, 1978a, 1978b; Larsson and Rehfeld, 1979) and peripheral nerves (Unwas-Wallen'ster et al., 1977; Larsson and Rehfeld, 1979). They are synthesized in neuronal tissue (Golterman et al., 1980) and concentrated in synaptic vesicles from where they are released like other neurotransmitters (Emson et al., 1980). Larsson and Rehfeld (1979) studied the distribution and molecular nature of CCK in peripheral nerves and observed the presence of CCK nerve terminals in pancreatic islets. The predominant form of CCK in these nerves seems to be the C-terminal tetrapeptide amide, Trp-Net-Asp-Phe-NH$_2$, common to CCK and gastrin, referred as CCK-4. The effect of various cholecystokinins and gastrins on the isolated and perfused pancreas showed that CCK-4 was considerably more effective in releasing insulin in man (Rehfeld, 1971) and in
mammals (Rehfeld et al., 1980). It has been also suggested that the secretion of insulin and other islet hormones is under neural control by the tetrapeptide amide (Rehfeld et al., 1980). CCK is closely related to gastrin both structurally and functionally and probably shares a common evolutionary origin (Larsson and Rehfeld, 1977). Their identical C-terminal sequence is responsible for their biological activity and the remaining parts modify their potency on different target tissues. The effect of different molecular forms of CCK on release of insulin and other islet hormone was studied and it was found that all CCKs stimulated the secretion of all pancreatic hormones in a dose dependent manner (Rehfeld et al., 1980) and it was shown that CCK-4 was the most potent peptide even at the lowest concentration $10^{-10}$ M, it was a powerful secretagogue. The intact tetrapeptide amide with free Trp- and Phe-NH$_2$ termini seemed necessary for the activity. Thus, synthetic peptides with NH$_2$ terminal modifications, such as removal of Trp (CCK-3), extension of glycine (CCK-5), gastrin pentapeptide or by BOC-$\beta$-alanine (Peptavlon) greatly reduced the secretagogue activity, C-terminal modification also reduced the potency. The deaminated tetrapeptide Trp-Met-Asp-Phe-OH stimulated insulin secretion at the highest concentration $10^{-6}$ M (Rehfeld et al., 1980). In this laboratory two analogues of CCK-4 have been synthesized replacing Trp, by p-glu and Pro at NH$_2$-terminals. Their secretory activities will be discussed.
WORKING MODEL FOR INSULIN RELEASE

The model shown in (Fig. 7) describes the possible steps linking specific stimulus to the release of insulin. In B-cells, a specific glucose receptor exists on plasma membrane which recognizes and binds with D-glucose and preferentially complexes with the \( \alpha \)-anomer of D-glucose. As a result of this interaction the adenyl cyclase system is activated and through an unknown mechanism the B-cell membrane becomes more permeable to calcium, resulting in the influx of ionic calcium into the cell. The resultant change in the ionic flux is associated with a change in membrane potential. The interaction of D-glucose with the receptor may be inhibited by such agents as alloxan, which probably destroys the receptor, or by activation of \( \alpha \)-adrenergic receptor with epinephrine. In B-cells, cAMP plays a modulating role of releasing bound calcium from the organelle. This modulating effect of cAMP can enhance glucose-induced release when agents such as glucagon bind with the receptor and increase adenyl cyclase activity. The ionic calcium in association with other factors initiates a contraction of the microtubular - microfilamentous system with a resultant displacement of columns of \( \beta \)-granules on to the cell surface, where they are released by emiocytosis. This immediate release of insulin would form the first phase of secretion. The second phase of the release would be due to stored and newly formed granules becoming associated with the microtubular-microfilamentous system and released.
Fig. 7 - Theoretical scheme showing interrelationship between nucleotides, Ca$^{2+}$ and glucose as they affect insulin release. "Active Ca$^{2+}$" is generally assumed to be cytosolic but in fact may represent different compartments of Ca$^{2+}$ varying in their action on the secretion process (Grech et al., 1976).
by emiocytosis. The membranous sac which encased the granule fuses with the plasma membrane and are subsequently pinched off by the microtubular-microfilamentous system and appear as microvesicles in the cytoplasm. The microvesicles either are returned to Golgi complex or fused with the lysosomes and are degraded.

DIABETOGENIC AGENTS

Alloxan

Alloxan (Fig. 8) is known to produce selective destruction of B-cells in the islets of Langerhans (Dunn et al., 1963). The presence of D-glucose and certain other insulin secretagogues, prior to or during alloxan exposure prevent deleterious action of alloxan both in vivo and in vitro (Zawalich and Beidler, 1973; Rossini et al., 1975; Bansal et al., 1980). However, α-D glucose provides more protection at lower concentration than β-D glucose. Other agents which protect B-cells against alloxan toxicity are superoxide dismutase, catalase and scavengers of hydroxylradical (Granqvist et al., 1979a), trace metals (Granqvist et al., 1979b) superoxidemutase, catalase and a metal chelator (Fischer and Hamburger, 1980a), dimethylurea (Fischer and Hamburger, 1980b) and thioredoxin and NADPH-thioredoxin reductase (Holmgren and Lykke-Andersen, 1980).
The exact mechanism of alloxan toxicity to B-cell and its protection by glucose is still not clear. Zawalich and Beidler (1973) have reported that primary site of action of alloxan may not be the glucose transport site but the glucose-receptor site on the B-cells and that glucose protects against alloxan damage by producing conformational changes in its membrane. Rossini et al. (1974) and McDaniel et al. (1976) have reported the presence of specific structure and sites on B-cell membrane. Reports from this laboratory show that the protection by D-glucose against alloxan toxicity viz. pro-insulin biosynthesis and the reversal of glucose protection by alloxan may be due to the displacement of glucose or alloxan by one another from a common receptor. Furthermore, the striking similarities in the stereospecific and chemical structures of alloxan and D-glucose may account for the apparently competitive type of interaction between the two substances at the common receptor site (Bansal et al., 1980).

**Streptozotocin**

Streptozotocin (SZ) is derived from *Streptomyces acromogenes* and has antibacterial, oncogenic and cytotoxic properties particularly to B-cell (Rakieten et al., 1976). The compound consists of 1-methyl 1-nitrosourea moiety linked to carbon-2 of D-glucose and exists as either of two anomers in the pyronose form (Fig. 8) (Herr et al., 1967) and has been
Fig. 6 - Structures of alloxan and streptozotocin.
shown to produce irreversible hyperglycemia in rats, dogs, mice and rhesus monkeys (Rakieten et al., 1963; Anson et al., 1967; Pilkin and Reynolds, 1970). Diabetes onset following intravenous streptozotocin follows a triphasic pattern with an early hyperglycemia (peaking at second hour) followed by hyperglycemia (most marked at the seventh hour) and finally by the twenty-fourth hour (Junod et al., 1967; Junod et al., 1969). The work of Junod et al. clearly assigns the permanent diabetogenic property of SZ to its B-cell cytotoxic action whereas the mechanism behind the early hyperglycemia phase is yet to be elucidated. The thymus-dependent functions play an obligatory etiologic role in the development of diabetic in mice treated with repeated subdiabetogenic doses of SZ (Paik et al., 1980). Furthermore, it has been suggested that glucose component of SZ enhances the uptake of SZ into islet cells, whereas the cytotoxicity of methylnitrosourea can exert its full effect (Gunnarsson et al., 1974). Glucose does not provide any protection against its diabetic action (Rerup, 1970), hence, SZ and glucose appear to have different combining sites on cell surface (Gonda et al., 1976). Rossini et al. (1977) have suggested that initial mediation of SZ toxicity is at a receptor site possibly on the surface of B-cell.

Schein et al. observed that nicotinamide a precursor of NAD $^+$ protects against the diabetogenic effect of SZ (Schein et al., 1967; Schein and Loftus, 1968; Schein and Bates, 1968).
Further studies have demonstrated that nicotinamide could be administered two hours after SZ without any loss of its protective effect (Dulin and Wyse, 1969a,b; Stauffacher et al., 1970). It was postulated that SZ acts on B-cell by depletion of NAD⁺ (Dulin and Wyse, 1969a). This notion has been supported by direct measurements that showed that SZ caused a very large decrease of islets NAD⁺ within 1 to 2 hour after the injection or after addition in vitro to isolated islets (Ho and Hashim, 1972; Henz et al., 1973 and Schein et al., 1973).

Rats injected with nicotinamide and SZ develop grossly visible tumours of pancreatic islets and using an i.v. glucose tolerance test, some tumour bearing animals exhibited a vigorous response to glucose loading, whereas other showed a subdiabetic response and tumour from both groups released immunoreactive insulin (Frigo et al., 1981). However, the effect of SZ on proinsulin biosynthesis still remains to be elucidated.

BINDING AND ACTION OF INSULIN

The biological effects of insulin embrace most of the vital functions necessary for development and growth. These range from rapid effects on membrane transport (Krahl, 1974; Pilkis and Park, 1974), activation and inhibition of both membrane bound and soluble enzymes of carbohydrates, lipid and protein metabolism (Krahl, 1972), alterations of cell morphology (Evans et al., 1974) to delayed effects on protein synthesis
(Manchester, 1970) and degradation (Mortimore and Mondon, 1970), accumulation of casein mRNA into mouse mammary epithelial cells (Franklyon, 1981), stimulation of the phosphorylation of ribosomal protein from 373-11 adipocyte increases glucose transfer across blood brain barrier (Hertz et al., 1981), stimulation Na-K ATPase from rat hippocampus (Berrstein et al., 1981) and stimulation of glucose incorporation and amino acid transport (Knight et al., 1981). Despite the fact that insulin has been for many years been available as a comparatively pure protein hormone, its primary site of action (if indeed there is a single primary site) is virtually unknown.

It is possible that insulin regulates all of its action via a putative second messenger distinct from cAMP but its identity remain unknown (Fritz and Lee, 1972). Thus insulin and its receptors seem to interact and remain in the extracellular or plasma membrane phase while the second messenger (X) relays the signal into cells interior (Fig. 9).
Fig. 9 - Scheme depicting possible insulin receptor transformations. Panel A shows the conventional model based on reversible external binding of insulin and its receptor. An alternative and equally attractive mechanism (Panel B) could be that the insulin induced dissociation of receptor subunits open a channel in the membrane through which the insulin monomer can pass into the cell (Steiner, '96a)
MATERIALS AND METHODS
The following materials were procured from the agencies mentioned against their names:

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
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<tbody>
<tr>
<td>Golden white mushroom (<em>Agaricus bisporus</em>)</td>
<td>National Botanical Research Institute, Lucknow, India</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Pharmacia Fine Chemicals Uppsala, Sweden</td>
</tr>
<tr>
<td>Sephadex G-100 and G-50</td>
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<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma Chemical Co., St. Louis, Mo, U.S.A.</td>
</tr>
<tr>
<td>Collagenase type V</td>
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<tr>
<td>Porcine insulin (23.6 IU/mg)</td>
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<tr>
<td>Streptozotocin</td>
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<tr>
<td>Aprotinin (Trasylol)</td>
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<tr>
<td>α-Methyl-D-glucoside</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>British Drug House, Poole, England</td>
</tr>
<tr>
<td>N,N'-methylene bis acrylamide</td>
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<tr>
<td>Material</td>
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<td>Ammonium persulphate</td>
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<td>Methylene blue</td>
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<tr>
<td>Nicotinamide</td>
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<td>$^{14}$C-leucine (sp. act. 20.8 mCi/mmole)</td>
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<td>$^{125}$I-insulin (sp. act. 100 mCi/µg of insulin)</td>
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<tr>
<td>$^{45}$Ca$^{2+}$- as CaCl$_2$ (sp. act. 4.0 µCi/g of Ca)</td>
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</tbody>
</table>

All other reagents and chemicals used were of analytical grade (AR).
ANIMALS

Animals used in the present studies were derived from animal colony of Central Drug Research Institute, Lucknow, housed in air-conditioned rooms. Charles Foster albino male rats maintained on standard balanced pellet (Hindustan Lever, Bombay, India) and water ad libitum were used throughout the experiments. Albino rabbits and guinea pigs fed animal house chow and water ad libitum were used.

ISOLATION, PURIFICATION AND CHARACTERIZATION OF AGARICUS BISPORUS LECTINS

Extraction of lectins

Golden white mushrooms (Agaricus bisporus) were homogenized in 0.9% NaCl (w/v) in a Waring Blender at room temperature as described in the modified method of Sage and Connell (1969). The homogenate was kept for 12 hours before the extraction of proteins at room temperature. The homogenized mixture was centrifuged at 10,000 rpm for 15 min, the supernatant fluid was heated at 60°C for 2 hours (temperature was maintained in a water bath incubator) and then centrifuged again at the same speed. The dark brown supernatant fluid (crude extract) was dialysed against 0.02 M phosphate buffer pH 7.2 and stored at -20°C.
Isolation of lectins by ConA sepharose affinity chromatography

Preparation of ConA sepharose

Sepharose 4B was activated by CNBr according to the procedure which was essentially similar to that of March et al. (1974). CNBr was synthesized in this laboratory according to the procedure described in Organic Synthesis, Vol. 2. A white crystalline product with melting point of 49-51°C and boiling point of 60-61°C was obtained. Crystalline CNBr was dissolved in acetonitrile (2 g CNBr/CH₃CN) and stored frozen in small aliquots. 10 ml of gel sepharose 4B was added to equal volume of 2 M sodium bicarbonate and mixed by stirring slowly. The rate of stirring was increased and 0.5 ml acetonitrile solution of CNBr was added all at once and the slurry was stirred vigorously for 2 minutes. The slurry was poured into a sintered glass funnel, washed with 5-10 volumes each of 0.1 M sodium bicarbonate, pH 9.5 and water. The slurry was filtered under vacuum to a moist, compact cake and transferred to a bottle containing 10 ml of 0.2 M sodium bicarbonate, pH 9.5. Coupling was done at 4°C according to the method described by Porath et al. (1967). 300 mg concanavalin A dissolved in 2 M sodium bicarbonate, pH 9.5, added to the activated gel and stirred for 36 hours. The reaction was stopped by adding 1 M glycine. After coupling the gels were washed alternatively with 0.1 M
acetate buffer, pH 4.0 and 0.1 M sodium bicarbonate, pH 9.5, several times. 11.8 mg Con A per ml gel was coupled.

**Preparation of phosphocellulose**

Phosphocellulose was prepared by the procedure which was essentially similar to that of Peterson and Sober (1961). 75 g of NaOH was dissolved in 185 ml of water and was stirred with 50 g of cellulose. The well mixed mass was kept in ice bath for 30 mins and 350 ml water was added slowly to the mixture. To this a mixture of 80 ml ether and 40 ml POCl₃ was slowly added with constant stirring at room temperature (25-30°C) so that the addition is completed in about 1 hour. The phosphocellulose suspension obtained was diluted to 2 liters with water, particles were washed repeatedly with water and filtered. It was again washed with water and ethanol and dried by evaporating the alcohol and water in a round bottom flask at reduced pressure.

**Column chromatography**

Con A sepharose was packed in a column (15 x 1.5 cm). It was washed with 1 M NaCl containing 1 mM MnCl₂, MgCl₂ and CaCl₂ each and finally equilibrated with 0.02 M phosphate buffered saline (PBS) pH 7.2. The dark brown crude lectin extract was passed through the column, washed with the same buffer and the unadsorbed fractions were discarded. The lectins were eluted with 0.1 M D(-methyl-D-glucoside in PBS. The hemagglutination
containing fractions were pooled, dialysed against the same buffer, lyophilized and reconstituted in a small amount of PBS. The concentrated lectin solution was dialysed against 0.005 M phosphate buffer pH 5.8 and then applied on phosphocellulose column (15 x 1.5 cm) pre-equilibrated with the same buffer. At this concentration and pH, 85% of the hemagglutination activity (PHA-A) was eluted and the remaining hemagglutination activity (PHA-B) was eluted after raising the pH of the buffer to 8.2. Both the fractions were pooled separately, dialysed, lyophilized and reconstituted in small amount of 0.02 M PBS, pH 7.2.

Isolation of Agaricus bisporus lectin using stroma as affinity adsorbent

Stroma was prepared as described by James et al. (1963). 80 ml of blood was collected from rabbit in anticoagulant, centrifuged and washed three times with 0.15 M PBS, pH 7.2 to get erythrocytes. 2 ml of the erythrocyte suspension was taken in each 40 ml polypropylene tube and 28 ml of 0.02 M phosphate buffer (hypotonic solution) was added to achieve lysis of the cells. It was then centrifuged at 18,000 rpm for 40 minutes. The membranes were washed with 0.02 M phosphate buffer pH 7.2 and resuspended in 0.1 N KOH and kept overnight. The suspension was recentrifuged at 18000 rpm for 40 minutes and the residue was freeze dried. 350 mg freeze dried stroma was mixed with 100 ml PBS and 8 ml 25% glutaraldehyde at pH 7.3 and was
incubated at 20°C for 16 hours. It was extensively washed with PBS by centrifugation and resuspended in 25 ml of the same buffer to which 1 M glycine was added to neutralize free -CHO groups. It was then kept at 20°C overnight. The solution was centrifuged and the stroma was washed again with PBS. It was then homogenized to make a suspension of fine particles in 25 ml PBS. 6 ml of swelled sephadex G-50 and 5 ml of stroma suspension were mixed and packed in a column.

The column was washed with water, glycine-HCl buffer pH 2.5 and finally with PBS. The sample was applied and eluted with water and the remaining activity was eluted with glycine-HCl buffer pH 2.5. The fractions eluted with water and glycine-HCl buffer agglutinated with rabbit erythrocytes. The yield was low.

Isolation of *Agaricus bisporus* lectin using formalinized erythrocytes

Method employed for the purification of the lectin was essentially similar to that of Neitherman et al. (1974) except that formalinized rabbit erythrocytes were used in the present method instead of formalinized sheep erythrocytes. 100 ml blood was collected in anticoagulant, spun down at 4000 rpm, washed three times with 0.15 M PBS, pH 7.2 and finally taken in 0.17 M PBS, pH 7.2 as 8% suspension. Equal volume of formalin (pH 7.2) was mixed with erythrocytes suspension and was shaken for 18 hours at a constant temperature of 37°C in a metabolic
shaker. The suspension was again centrifuged, the residue (erythrocytes) was washed once with 2.5 volumes of the 0.15 M PBS pH 7.2 and stored at 4°C. Crude extract of *Agaricus bisporus* was incubated with formalinized erythrocytes for 30 minutes at room temperature. It was then centrifuged and the supernatant was discarded. The sediment was suspended in 0.1 M lactose in PBS, for 30 minutes and recentrifuged. Agglutination was noticed after removing lactose from the solution.

**HEMAGGLUTINATION ASSAY**

Hemagglutination assays were performed by using 2% rabbit erythrocytes suspension by double fold dilution method on glass slides, as described by Ochoa and Krishtainsen (1978). 0.1 ml of 2% erythrocyte suspension was mixed with 0.1 ml of lectin solution on glass slides. Agglutination was seen after one hour.

**PROTEIN ESTIMATION**

Protein in eluants was monitored by absorption at 280 nm in a Beckman DU spectrophotometer model-24 and estimated according to Lowry et al. (1951) method using bovine serum albumin (BSA) as standard.
POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was conducted using 7% gel in a tris glycine buffer (Davis, 1964), pH 8.9 and subjected to electrophoresis at 4 mA per gel tube using a Broviga, Madras, India, electrophoresis system. The gels were stained with 1% coomassie blue into methanol : acetic acid : water (5:1:5, v/v) and destained by 7% acetic acid.

SUGAR ESTIMATION

Neutral sugars were estimated by phenol sulphuric acid method as described by Dubois et al. (1956), using glucose as standard.

MOLECULAR WEIGHT DETERMINATION

Molecular weight of pure A. bisporus PHA-A and PHA-B was determined by gel filtration. A sephadex G-100 column (1.0 x 100 cm) was pre-equilibrated with 0.02 M phosphate buffer, pH 7.2 and calibrated with aldolase (145,000), BSA (68,000), trypsin (23,300) and lysozyme (17,500) as known molecular weight protein markers. The ratio of elution volume (Ve) and void volume (Vo) was plotted against \( \log_e \) molecular weight.
ISOLATION OF ISLETS OF LANGERHANS FROM RAT PANCREAS

The islets of Langerhans were isolated according to the procedure of Iacy et al. (1967) as modified by Feldman and Chapman (1975). The pancreatic islets were stimulated by intraperitoneal injection of pilocarpine (4% w/v) prior to sodium pentabarbital (0.6% w/v). The pancreas was quickly removed, cleaned off the extraneous adipose tissue and chopped into small pieces in Hanks solution. The fine pieces were washed several times with Hanks solution following removal of fats. Collagenase (40 mg/500 g body weight) was added to the fine pieces in 3 ml Hanks solution and stirred in a metabolic shaker for 20–30 minutes at 37°C. The islets were picked up by means of pasteur pipette under a dissecting microscope and transferred in KRB medium.

OXYGEN UPTAKE BY ISOLATED ISLETS OF LANGERHANS

Oxygen uptake by islets was studied by Warburg manometric technique. All the glasswares were siliconized. 10–15 islets in 2.6 ml KRB medium were placed in the side arm of each Warburg flask and 0.2 ml KOH was taken in the central well. In the control flask 2.6 ml KRB containing 2 mM glucose was taken while the two experimental flasks contained 2 mM glucose + 17.5 μg/ml Agaricus bisporus lectin in 2.6 ml KRB (this concentration of the lectin produces half maximal stimulation of
insulin release). The temperature of the flasks were equili-
berated at 37°C for 15 minutes. The contents were then mixed
and the readings were taken at 10 min interval for 2 hours.
The detail of the apparatus is described by Burk and Milner
(1932), Dexon (1951), Perkins (1943), Warburg (1923, 1924, 1926)
and others.

INCUBATION OF ISOLATED ISLETS OF LANGERHANS WITH
AGARICUS BISPORUS LECTIN

All the glasswares were siliconized. Groups of five
islets were incubated in 1 ml of Krebs Ringer bicarbonate medium
(Gey and Gey, 1936) containing 2 mM Ca++, 3 mg/ml of bovine
serum albumin, 12 naturally occurring amino acids (Eagle, 1959),
0.01 M non-essential amino acid, 2 mM glucose and various con-
centrations of Agaricus bisporus lectin (17.5 µg/ml to 100 µg/ml).
The gas phase was O₂ + CO₂ (95 : 5) and the pH was 7.4. The
incubation was carried out for 3 hour at 37°C in a metabolic
shaker (72 oscillations per minute). Samples were drawn at an
interval of 1 hour for the estimation of their insulin content
by radioimmunoassay. For the protection of insulin against
proteolytic degradation, trasylol was added in the incubation
medium to give final concentration of 1,000 KIU per ml and the
samples were stored at -80° till the insulin assay was
performed.
Radioimmunoassay technique used was the modified method of Morgan and Lazarow (1963) as described below.

**First antibody or anti-insulin guinea pig serum**

It was obtained by immunizing guinea pigs against bovine insulin (6 times crystallized). Equal volumes of insulin solution 6-12 IU/ml and complete Freund's adjuvant (CFA) were emulsified and administrated to guinea pigs subcutaneously at one week interval for four weeks. Then after 10 days interval two booster doses were administered. The animals were bled after 7th-10th day of the last injection when the antibody titer was maximum.

**Second antibody or anti-guinea pig gama globulin rabbit serum**

It was obtained by immunizing rabbits against guinea pig gama globulin. 1 ml (10 mg/ml) guinea pig gama globulin emulsified in 1 ml of CFA were administered subcutaneously to rabbit every week for four weeks. The two booster doses were administered after 10 day interval. The animal was bled after 7th-10th day of the last injection.

**Method**

0.5 ml borate buffer, 0.05 M, pH 8.6 was mixed with 0.1 ml of standard insulin or unknown samples and 0.1 ml of
first antibody (1 : 1,00000 dilution) and kept overnight at 4°C. 0.1 ml of $^{125}$I-insulin was then added to the sample mixture and was again kept overnight at 4°C. After that 0.1 ml of second antibody (1 : 60-100 dilution), and 0.1 ml normal guinea pig serum(NPGS) (1 : 5000 dilution) was added and again kept overnight at 4°C. It was then centrifuged at 4000 rpm for 20 min and the radioactive counts in the residues were taken in a Packard gama scintillation spectrometer. This gives the binding. Dose response curve was made by plotting insulin concentration against % binding. The results are expressed as $\mu$U/ml or islets.

**UPTAKE OF $^{45}$Ca$^{2+}$ BY ISLETS IN PRESENCE OF GLUCOSE AND AGARICUS BISPORUS LECTIN**

In 25 ml conical flasks batches of five islets were taken in 1 ml KRB containing BSA 3 mg/ml, 12 naturally occurring amino acids according to Eagle (1959), non-essential amino acid 0.01 M, trasylol 1000 KIU and glucose 2 mM. Different concentrations of A. bisporus lectins (17.5 $\mu$g/ml-100 $\mu$g/ml) and 5 $\mu$Ci $^{45}$Ca$^{2+}$ were added to make the final volume of 1 ml. In the control lectin was omitted. The reaction was carried out for two hours at 37°C in a metabolic shaker. The pH of the medium was 7.4. For time courses studies 2 mM glucose + 17.5 $\mu$g/ml lectin was incubated with islet and 2 mM glucose alone was taken as control. The reaction was stopped after 10 min by adding 2 mM lanthanum.
chloride. The incubations were continued for two hours with the same experimental conditions. Samples for insulin assay were taken. The incubation was followed by 1 hour of washing with non-radioactive basal medium, supplemented with 2 mM lanthanum chloride according to the method of Hellman et al. (1976). The islets were spun down and washed with LaCl$_3$ (2 mM). The islets were then ruptured in 0.1% triton-x followed by freeze and thawing (in liquid air). Suitable aliquotes were added to 10 ml scintillation fluid (0.4% 2,5-diphenyl oxazole and 0.01% 1,4 bis (5-phenyl oxazolyl-2) in toluene and 2-methoxy ethanol (1:1 v/v) and radioactive counts were taken in a Packard Tricarb Liquid Scintillation Spectrometer, model 3330. The results are expressed as cpm/5 islets.

INCORPORATION OF $^{14}$C-LEUCINE INTO ISLET PROTEINS

Incorporation of $^{14}$C-leucine into islet protein was carried out in KRB medium containing bovine serum albumin and 12 naturally occurring amino acids according to Eagle (1959) except that leucine was omitted. The concentration of non-essential amino acids in the medium was 0.01 M (basic medium). The incubation was carried out at 37°C in a metabolic shaker (72 oscillation per minute) in an atmosphere of 95% O$_2$ and 5% CO$_2$. The pH of the medium was maintained at 7.4.

For in vitro studies, 24 hours fasted rats were used. Batches of 60-70 islets from the experimental rats were incubated
in 1 ml of KREB medium for 30 min (preincubation). The medium
was then removed and islets were further incubated for 3 hours
in 1 ml of KREB containing 2 mg/ml BSA and 3 mg/ml glucose,
10 μCi of 14C-leucine (sp. act. 20.8 mCi/mmole) and streptozotocin
at concentrations 0.05 mg-1.5 mg per batch. In other set of
experiments under similar conditions described above, nicotinamideme 1 mg-4 mg was soon added to SZ (1.5 mg) (keeping SZ constant
in all sets) for protective studies. In one set 17.5 μg/ml of
A. bisporus PHA-B, was added in the presence of glucose. The
islets were rapidly washed 3 times with 2 ml of medium containing
cold leucine (5 mM). Adherent tracer was removed from the
islets by incubating in 1 ml basic medium containing cold
leucine (0.2 mM) for 10 minutes.

EXTRACTION OF ISLET PROTEINS

Since it is known that no significant release of pro-
insulin takes place before 200 min of incubation (Stoever and
Oyer, 1967), the above incubation medium in which the islets
were incubated for a maximum period of 3 hours was discarded.
The islets were ruptured by freeze and thawing (in liquid air)
following homogenization in cold TCA (10%, w/v) centrifuged
and extracted twice with acid-ethanol (ethanol: 0-phosphoric
acid: water; 89:1:19, v/v) for a total period of 20 hours
(Davoren, 1962). The two extracts were pooled and residue was
dissolved in 1 N sodium hydroxide. Suitable aliquotes of both
the fractions were added to scintillation vial containing 10 ml of scintillation fluid (0.4% PPO and 0.01% POPOP in toluene: 2-methoxy ethanol; 1:1 v/v) and counted in Packard Tricarb liquid Scintillation Spectrometer, model 3330. The radioactive counts in the extract were taken as an index of \(^{14}\)C-leucine incorporation into proinsulin fraction, while incorporation into total islet proteins was determined by adding to it the counts in the residue. The results are expressed as cpm/mg islet protein.

INCUBATION OF CCK-4 TETRAPEPTIDES WITH ISLETS OF LANGERHANS

Group of 5 islets were incubated with 1 ml KRB (containing 3 mg/ml BSA, 12 naturally occurring amino acid according to Eagle (1959), 0.01 M non-essential amino acids, 1000 KIU/ml trasylol, and 2 mM glucose) and the CCK-4 tetrapeptides in the concentration range of \(10^{-10}\), \(10^{-8}\) and \(10^{-6}\) M each. Similarly as in other insulin secretion studies, the incubations were carried out at 37°C in a metabolic shaker at pH 7.4 for 3 hours. Samples were drawn from the media at an interval of 1 hour for the estimation of insulin by radioimmunoassay.
RESULTS
The results of the purification of Agaricus bisporus PHA at various steps are summarized in Table 2. Agaricus bisporus PHA was purified with a recovery of 90.8% and 6-fold purification by ConA sepharose column chromatography (Fig. 10). Crude extract (NaCl extract, heat treated) was passed through ConA sepharose column as described in Materials and Methods. On heat treatment most of the proteins got denatured.

The preparation was resolved into two hemagglutinating fractions by phosphocellulose column chromatography. Thus, about 80% of the hemagglutinating activity of the ConA sepharose eluate was eluted at pH 5.85 (Fraction PHA-A), whereas 15% of the hemagglutinating activity was released from the column when the pH of the eluting buffer was raised to pH 8.2 (Fraction PHA-B) (Fig. 11).

The polyacrylamide gel electrophoresis of the ConA sepharose eluate (PHA) exhibited one major and one minor band.
Table 2 - Purification of lectins from *Agaricus bisporus*.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity HAU b</th>
<th>Specific activity b/a</th>
<th>% Recovery (in terms of activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Crude supernatant (NaCl extract, heat treated)</td>
<td>30</td>
<td>19.90</td>
<td>19200</td>
<td>964.80</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Con A sepharose column chromatography</td>
<td>4</td>
<td>3.30</td>
<td>17440</td>
<td>5406.06</td>
<td>90.80</td>
</tr>
<tr>
<td>3.</td>
<td>Phosphocellulose column chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a) PHA-A eluted at pH 5.8</td>
<td>3</td>
<td>2.50</td>
<td>13962</td>
<td>5584.50</td>
<td>80.00</td>
</tr>
<tr>
<td></td>
<td>(b) PHA-B eluted at pH 8.2</td>
<td>3</td>
<td>0.465</td>
<td>2616</td>
<td>5625.80</td>
<td>15.00</td>
</tr>
</tbody>
</table>
Fig. 10 - Con A - sepharose affinity chromatography of *Agaricus bisporus* lectin (crude extract) (Flow rate 20 ml/hr and fraction volume 4 ml).

(●-----●) Absorbance at 280 nm;

(○-----○) Hemagglutination unit (HAU)
Fig. 11 - Phosphocellulose chromatography of Con A-sepharose eluate. Flow rate 10 ml/hr and fraction volume 4 ml).

(o-o) Absorbance at 280 nm;
(e-e-e) Hemagglutination unit (HAU)
corresponding to PHA-A and PHA-B (Fig. 12). When passed through phosphocellulose column the PHA could be separated into two fractions, PHA-A and PHA-B, both fractions when eluted exhibited single band separately on polyacrylamide gel electrophoresis (Fig. 12), showing homogeneity of the two PHAs fractions. Neutral sugars content of the purified PHA was found to be 2.5% as estimated by phenol sulphuric acid method.

The molecular weight of the purified Agaricus bisporus PHA-A and PHA-B were determined using sephadex G-100 gel filtration. The ratio of elution volume (Ve) to void volume (Vo) was plotted against log molecular weight (log MW) for the marker proteins aldolase (145,000), BSA (68,000), trypsin (23,300) and lysozyme (17,500) (Fig. 13). PHA-A had an elution volume corresponding to the molecular weight of 64,100 whereas PHA-B had an elution volume corresponding to the molecular weight of 58,000.

EFFECT OF AGARICUS BISPORUS PHA-B ON OXYGEN UPTAKE BY ISOLATED ISLETS OF LANGERHANS

Agaricus bisporus PHA-A does not stimulate hormone release from the islets. These experiments were carried out in order to study the effect of Agaricus bisporus PHA-B (the insulin releasing fraction) on O₂ uptake by the islets of Langerhans under the in vitro conditions of our studies. The results of
Fig. 12 - Polyacrylamide gel electrophoresis of (a) Con A-Sepharose eluate; (b) PHA-A and (c) PHA-B. 7.5% gel was used in 0.1 N tris glycine buffer at pH 8.9.
Fig. 13 - Determination of molecular weight by exclusion chromatography on sephadex G-100 of *A. bioporum*, PHA-A and PHA-B. The ratio of elution volume to void volume ($V_e/V_0$) was plotted against log mol. wt for the marker proteins aldolase (145000), BSA (68,000), trypsin (23,300) and lysozyme (17,500). The arrows indicate the mol. wts of PHA-A and PHA-B.
the effect of glucose (2 mM), glucose (20 mM), glucose (2 mM) + \textit{A. bisporus} PHA-B (17.5 µg/ml) and glucose (20 mM) + \textit{A. bisporus} PHA-B (17.5 µg/ml) on the isolated islets of Langerhans, with respect to the O\textsubscript{2} uptake are shown in Table 3.

It can be seen that in the presence of 2 mM glucose alone and 2 mM glucose + 17.5 µg/ml \textit{A. bisporus} PHA-B (that produces half maximal stimulation of insulin release) µl of O\textsubscript{2} uptake are 3.83 and 3.9 respectively. It is evident that PHA-B has no effect on O\textsubscript{2} uptake by islets and therefore does not seem to alter the rate of islet glucose metabolism.

Similarly no significant change in O\textsubscript{2} uptake was observed when the glucose concentration was raised to 20 mM keeping the PHA-B concentration at 17.5 µg/ml. The results are presented in Table 3.

\textbf{IN VITRO STUDIES ON AGARICUS BISPORUS PHA-B MEDIATED INSULIN RELEASE AND \textsuperscript{45}Ca\textsuperscript{2+} UPTAKE BY ISLETS OF LANGERHANS}

\textit{Agaricus bisporus} PHA-B and \textit{Agaricus campestris} PHA\textsubscript{i} are the two lectins known, which stimulate insulin release from isolated islets of Langerhans in the presence of sub-stimulatory concentration of glucose (2 mM) in the medium. The effect of the purified \textit{A. bisporus} PHA-B on islets of Langerhans of rat pancreas with respect to insulin release and \textsuperscript{45}Ca\textsuperscript{2+} uptake has been studied and the results are presented as follows.
Table 3 - Glucose metabolism to CO$_2$ (O$_2$ uptake) in the absence and presence of A. bisporus PHA-B by isolated islets of Langerhans of rat pancreas. 17.5 µg/ml lectin was used in each experiment (That produces half maximal stimulation of insulin release). Batches of 10-15 islets were taken for each experiment.

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Glucose oxidation to CO$_2$ µl of O$_2$/islet/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glucose (2 mM)</td>
<td>3.83</td>
</tr>
<tr>
<td>2. Glucose (2 mM) +</td>
<td></td>
</tr>
<tr>
<td>A. bisporus PHA-B (17.5 µg/ml)</td>
<td>3.90</td>
</tr>
<tr>
<td>3. Glucose (20 mM)</td>
<td>3.40</td>
</tr>
<tr>
<td>4. Glucose (20 mM) +</td>
<td></td>
</tr>
<tr>
<td>A. bisporus PHA-B (17.5 µg/ml)</td>
<td>3.20</td>
</tr>
</tbody>
</table>
Effect of *A. bisporus* PHA-B on insulin release from isolated islets of Langerhans

Of the two fractions of *Agaricus bisporus* PHA, only PHA-B has been found to stimulate insulin release. Its effect on the insulin release from isolated islets of Langerhans in vitro in the presence of sub-stimulatory concentration of glucose (2 mM) has been studied and the results are shown in Table 4. It can be seen that at 17.5 µg/ml lectin concentration the insulin release is 109 ± 10 µU/5 islets/hr as compared to the control (2 mM glucose alone), which is 33 ± 5 µU/5 islets/hr. Thus there is 3.3 fold increase in insulin release in response to *A. bisporus* PHA-B (17.5 µg/ml) as compared to the control. When the lectin concentration is raised to 50 µg/ml, the corresponding insulin release increases more than five folds as compared to the control.

Effect of varying concentrations of *A. bisporus* PHA-B on insulin release from isolated islets of Langerhans

The stimulatory effects of varying concentrations of *A. bisporus* PHA-B (17.5 µg/ml to 100 µg/ml) on insulin release from isolated islets of Langerhans are shown in Fig. 14. It can be seen that *A. bisporus* PHA-B mediated insulin release in dose dependent and at the lectin concentration of about 60 µg/ml the insulin release is maximum after which the release of insulin tends to be stationary. Thus the lectins mediated insulin
Table 4 - Effect of glucose and *A. bisporus* PHA-B on insulin release from isolated islets of Langerhans. Flask containing 5 islets were incubated for 1 hr in 1 ml KKB medium containing 2 mM glucose and 2 mM glucose + lectin concentration. Insulin release was measured by radioimmunoassay. Each result is expressed as mean rate of hormone release observed in six individual incubation; $\mu$U/5 islets/hr $\pm$ SD.

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>Lectin concentration ($\mu$g/ml)</th>
<th>Rate of insulin release ($\mu$U/5 islets/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM</td>
<td>-</td>
<td>33 $\pm$ 5</td>
</tr>
<tr>
<td>2 mM</td>
<td>17.5</td>
<td>109 $\pm$ 10</td>
</tr>
<tr>
<td>2 mM</td>
<td>50.0</td>
<td>165 $\pm$ 10</td>
</tr>
</tbody>
</table>
Fig. 14 - Effect of varying concentrations of *A. bisporus* PHA-B on insulin release from isolated islets of Langerhans. Each result is expressed as the mean of the rate of insulin release in terms of μU/5 islets/hr ± S.D. for six different experiments.
release seems to be a function of the lectin concentration.

Time course studies on basal and *A. bisporus* PHA-B stimulated insulin release from isolated islets of Langerhans

Lectin stimulated insulin release from isolated islets of Langerhans proceeds as linear function of time for about one hour. The results are shown in Fig. 15. The effect of 2 mM glucose (control) and 2 mM glucose + 17.5 μg/ml *A. bisporus* PHA-B on insulin release are presented. It can be seen that the lectin mediated insulin release is enhanced from the beginning. After 20 minutes of incubation the release of insulin in the presence of lectin is about three times as compared to the control. More or less the same proportion is maintained throughout the one hour period of incubation.

**45Ca**\(^{2+}\) uptake in the presence of glucose and *A. bisporus* PHA-B by islets of Langerhans

The results of the effects of glucose (2 mM) and glucose (2 mM) + *A. bisporus* PHA-B (17.5 μg/ml) on **45Ca**\(^{2+}\) uptake by isolated islets of Langerhans of rat are shown in Table 5. It can be seen that the **45Ca**\(^{2+}\) uptake by islets in the presence of glucose (2 mM) + *A. bisporus* PHA-B (17.5 μg/ml) is enhanced more than eleven folds as compared to the control. The cpm/5 islets after one hour of incubation with *A. bisporus* PHA-B...
Fig. 15 - Time course studies on basal (2 mM glucose and 17.5 μg/ml A. bisporus PHA-B stimulated insulin release from islets of Langerhans. Each result is expressed as the mean rate of insulin release in terms of μU/5 islets ± 3 D. for six different experiments.

0 = 2 mM glucose
* = 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B
Table 5 - Effect of glucose and A. bisporus PHA-B on $^{45}$Ca$^{2+}$ uptake by isolated islets of Langerhans. The islets were incubated with glucose, PHA-B and $^{45}$Ca$^{2+}$ for 1 hr and 2 hr followed by 1 hr washing with 2 mM Lanthamum. The results are presented as cpm/5 islet ± S.D. for 5 different experiments.

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>Lectin concentration (µg/ml)</th>
<th>$^{45}$Ca$^{2+}$ uptake (cpm/5 islets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM</td>
<td>-</td>
<td>1330 ± 12</td>
</tr>
<tr>
<td>2 mM</td>
<td>17.5</td>
<td>14052 ± 588</td>
</tr>
<tr>
<td>2 mM</td>
<td>50.0</td>
<td>42977 ± 202</td>
</tr>
</tbody>
</table>
(17.5 µg/ml) is 14052 ± 588 as compared to the control (2 mM glucose) which is 1330 ± 12. When the lectin concentration is increased to 50 µg/ml, the corresponding $^{45}\text{Ca}^{2+}$ uptake in terms of cpm increases to more than 30 folds as compared to the control.

Effect of varying concentrations of A. bisporus PHA-B on $^{45}\text{Ca}^{2+}$ uptake by islets of Langerhans

The results of the effects of various concentrations of A. bisporus PHA-B on $^{45}\text{Ca}^{2+}$ uptake by isolated islets of Langerhans are shown in Fig. 16. The results clearly demonstrate that the $^{45}\text{Ca}^{2+}$ uptake is dose dependent in the range of 25 µg/ml to 100 µg/ml. The rate of $^{45}\text{Ca}^{2+}$ uptake increases with the concentration of the lectin and the maximal uptake is achieved at lectin concentration of about 60 µg/ml. After that the rate of $^{45}\text{Ca}^{2+}$ uptake slows down till it reaches a stationary phase at about 75 µg/ml lectin concentration.

Time course studies of $^{45}\text{Ca}^{2+}$ uptake by islets of Langerhans in the presence of glucose and A. bisporus PHA-B

Islets were incubated with $^{45}\text{Ca}^{2+}$, 2 mM glucose and 2 mM glucose + 17.5 µg/ml lectin for various periods of time followed by 60 minutes of washing with lanthanum chloride (2 mM). The $^{45}\text{Ca}^{2+}$ uptake in the presence of 2 mM glucose alone as measured
Fig. 16 - Effect of varying concentrations of \textit{A. bispora} PHA-B on $^{45}$Ca$^{2+}$ uptake by isolated islets of Langerhans. Each result is expressed as the mean of the rate of $^{45}$Ca$^{2+}$ uptake in terms of cpm/5 islets ± S.D. for six different experiments.

- $\bigcirc$ = 1 hr incubation
- $\bullet$ = 2 hr incubation
after every 20 minutes interval for 180 minutes are shown in Fig. 17. It can be seen that the rate of $^{45}\text{Ca}^{2+}$ uptake increase linearly with time for about 120 minutes after which it becomes stationary. When A. bisporus PHA-B was added there was a significant stimulation in the uptake of $^{45}\text{Ca}^{2+}$ as evident from Fig. 17. Thus at 17.5 $\mu$g/ml lectin concentration the stimulation of $^{45}\text{Ca}^{2+}$ uptake in terms of cpm is $25 \times 10^3$ as compared to $5 \times 10^3$ in case of the control, after 2 hour of incubation.

**INSULIN RELEASE IN AGING IN RESPONSE TO AGARICUS BISPORUS LECTIN**

The effect of A. bisporus PHA-B on insulin release and $^{45}\text{Ca}^{2+}$ uptake in islets of Langerhans isolated from different age groups of rats have been studied and the results are presented as follows.

**Effect of glucose and A. bisporus PHA-B on insulin release from islets of Langerhans of different age groups of rats**

Islets from 1, 3, 6 and 12 month old rats were isolated and 5 islets of equal size from each group were incubated with 2 mM glucose + 17.5 $\mu$g/ml A. bisporus PHA-B, 2 mM glucose alone was taken as control. The results are presented in Fig. 18. It can be seen that the basal insulin release in the presence
Fig. 18 - Effect of glucose and A. bisporus PHA-B on insulin release from islets isolated from 1, 3, 6 and 12 month old rats. Each result is expressed as the mean rate of insulin release in terms of μU/5 islets/hr ± S.D. for six different experiments.

0 = 2 mM glucose
● = 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B
Fig. 17 - Time course studies of $^{45}$Ca$^{2+}$ uptake by isolated islets of Langerhans in the presence of 2 mM glucose and 2 mM glucose + 17.5 µg/ml A. bisporus PHA-B. Each result is expressed as the mean of $^{45}$Ca$^{2+}$ uptake in terms of cpm/5 islets ± S.D. for six different experiments.

0 = 2 mM glucose
• = 2 mM glucose + 17.5 µg/ml A. bisporus PHA-B
of 2 mM glucose was almost the same in all the group of islets irrespective of the age of rats.

It is evident that *A. bisporus* PHA-B stimulates insulin release quite significantly in younger animals. The effect of lectin on the islets decreases steadily with the age of animal. Thus, while the increase in the release of insulin in the presence of lectin in case of islets of one month old rat is 4-fold as compared to the control. Whereas the increase in insulin release in the presence of lectin is 2.7 fold as compared to the control in the case of islets of 12 month old rats. Fig. 18, also shows that there is a 1.5 fold increase in insulin release in the case of 1 month old rat as compared to 12 month old rat in response to lectin (*A. bisporus* PHA-B).

$^{45}$Ca$^{2+}$ uptake by islets of different age groups in response to glucose and *A. bisporus* lectin (PHA-B)

Glucose stimulates insulin release by stimulating the uptake of extracellular calcium. As has been shown in Fig. 18, the basal insulin secretion by islets with 2 mM glucose is not affected by the age of the animals. The corresponding $^{45}$Ca$^{2+}$ uptake studies were carried out and the results of the effect of 2 mM glucose on $^{45}$Ca$^{2+}$ uptake by islets of Langerhans isolated from 1, 3, 6 and 12 month old rats are shown in Fig. 19. It is evident that, like insulin secretion, the $^{45}$Ca$^{2+}$ uptake by islets in response to 2 mM glucose also does not alter with age.
Effect of glucose (2 mM) on $^{45}\text{Ca}^{2+}$ uptake by islets isolated from 1, 3, 6 and 12 month old rats. Each result is expressed as the mean of the rate of $^{45}\text{Ca}^{2+}$ uptake in terms of cpm/5 islets ± S.D. for six different experiments.

- $\square$ = 2 mM glucose (1 hr incubation)
- $\blacksquare$ = 2 mM glucose (2 hr incubation)
When the incubation period was increased from 1 hour to 2 hour there was no change in $^{45}\text{Ca}^{2+}$ uptake in islets of all age group (Fig. 20). Thus, at sub-stimulatory concentration of glucose (2 mM), the insulin secretion (Fig. 18) and $^{45}\text{Ca}^{2+}$ uptake by islets (Fig. 19) were almost the same irrespective of the age of the rats.

The results of the effect of A. bisporus PHA-B (17.5 $\mu$g/ml) on islets of 1, 3, 6 and 12 month old rats with respect to $^{45}\text{Ca}^{2+}$ uptake are presented in Fig. 20. It is evident from the plot that the $^{45}\text{Ca}^{2+}$ uptake by islets in the presence of lectin is significantly more in the case of islets isolated from younger rats than the islets isolated from the older ones. The increase in $^{45}\text{Ca}^{2+}$ uptake in response to the lectin is 54.5 fold in the islets of 1 month old rat as compared to the control when incubated for one hour, whereas the increase in $^{45}\text{Ca}^{2+}$ uptake is 20.4 fold in the case of the islets of 12 month old rats as compared to the control when incubated for one hour. The increase in the $^{45}\text{Ca}^{2+}$ uptake in response to lectin in the case of islets from 1 month old rat is 3-fold, when compared to the islets of 12 month old rats. The response of islets to lectin with respect to $^{45}\text{Ca}^{2+}$ uptake decreases steadily with age of the animals. When the incubation period is increased from 1 hour to 2 hour there is an upward shift in the graph (Fig. 20) but the effect of age remains the same.
Fig. 20 - Effect of *A. bisporus* PHA-B (17.5 μg/ml) on $^{45}\text{Ca}^{2+}$ uptake by islets isolated from 1, 3, 6 and 12 month old rats. Each result is expressed as the mean of the rate of $^{45}\text{Ca}^{2+}$ uptake in terms of cpm/5 islets.

- ○ = 2 mM glucose + 17.5 μg/ml *A. bisporus* PHA-B (1 hr incubation).
- ● = 2 mM glucose + 17.5 μg/ml *A. bisporus* PHA-B (2 hr incubation).
- △ = 2 mM glucose (2 hr incubation)
Streptozotocin is a known diabetogenic agent whose toxic action can be reversed by nicotinamide. The results of the study of the effect of streptozotocin nicotinamide interaction on proinsulin biosynthesis are presented as follows.

Effect of varying concentrations of streptozotocin on total islet protein biosynthesis

The results of the effect of varying concentrations of streptozotocin (0.05-1.5 mg) on the incorporation of $^{14}C$-leucine into total islet proteins are shown in Fig. 21. It can be seen that by increasing the concentration of streptozotocin from 0.05 to 1.5 mg per 60-70 islets, the biosynthesis of the total islet protein decreases correspondingly as compared to the control (non-streptozotocin exposed islets) as shown by $^{14}C$-leucine incorporation. Glucose stimulates total islet proteins biosynthesis including that of proinsulin which is a predominant protein of islets. Streptozotocin concentration of 1.5 mg per 60-70 islets, causes the maximum inhibition of the incorporation of $^{14}C$-leucine into the islet proteins. It can be seen (Fig. 21), that at the streptozotocin concentration of 1.5 mg the islet protein biosynthesis is diminished by 5.5 fold as compared to the control.
Fig. 21 - Effect of ST inhibition on the incorporation of $^{14}$C-leucine into total islet proteins. Following a 30 minute preincubation the islets were exposed to ST at different concentrations in the presence of 5 mg/ml glucose. Each result is expressed as the mean of $^{14}$C-incorporation in terms of cpm/mg islet proteins ± S.D. for six different experiments.

□ = Proinsulin-insulin fractions
■ = Other proteins
Effect of varying concentrations of nicotinamide on streptozotocin toxicity

The results of the effect of varying concentrations of nicotinamide (0.5 mg-4 mg per 60-70 islets) on the inhibitory action of streptozotocin, as determined by the incorporation of $^{14}$C-leucine into the total islet proteins are shown in Fig. 22. Keeping the concentration of streptozotocin constant (1.5 mg) that causes maximum inhibition of $^{14}$C-leucine incorporation, the effect of increasing concentrations of nicotinamide (0.5-4 mg) show that with higher concentration of nicotinamide the inhibitory effect of SZ is reversed, and at the nicotinamide concentration of 4 mg in the medium, the protein biosynthesis is restored to the control value (non-streptozotocin exposed islets).

Effect of varying concentrations of streptozotocin on proinsulin biosynthesis

Glucose stimulates the proinsulin biosynthesis in preference to other islet proteins. The results of the incorporation of $^{14}$C-leucine into acid ethanol extractable proteins (proinsulin) of the islets which were exposed to streptozotocin in vitro at different concentrations (0.05 mg-1.5 mg) are presented in Fig. 23 and Fig. 24 in terms of % inhibition and % proinsulin biosynthesis respectively. It can be seen that addition of 1.5 mg of streptozotocin per 60-70 islets, in the
Fig. 22 - Effect of varying concentrations of nicotinamide on S2 inhibitory action of the \textsuperscript{14}C-leucine incorporation into total islet proteins. Each result is expressed as the mean of \textsuperscript{14}C-leucine incorporation in terms of cpm/mg islet proteins $\pm$ S.D. for six different experiments.

$\Box$ = Pro-insulin-insulin fraction

$\blacksquare$ = Other proteins
Fig. 23 - Streptozotocin inhibition (%) on subsequent glucose induced proinsulin biosynthesis. Each result is expressed as the mean of % inhibitions ± S.D. for six different experiments.
Fig. 24 - Retardation of (%) proinsulin biosynthesis at different concentrations of streptozotocin. Each result is expressed as the mean of the biosynthesis for six different experiments.
medium results in 79% inhibition of the incorporation of the tracer into acid ethanol extractable proteins which predominantly consists of proinsulin. At this concentration the proinsulin biosynthesis is only 21.5% as compared to the control (non-streptozotocin exposed islets) (Fig. 24).

Effect of varying concentrations of nicotinamide on the inhibition of proinsulin biosynthesis by streptozotocin

The results of the effect of different concentrations of nicotinamide on the streptozotocin inhibited incorporation of $^{14}$C-leucine into proinsulin are shown in Fig. 25. The concentration of streptozotocin was fixed at 1.5 mg (that causes 79% inhibition of proinsulin biosynthesis) whereas the concentration of nicotinamide was increased from 0.5 mg to 4 mg per 60-70 islets. At nicotinamide concentration of 4 mg per 60-70 islets there is 100% protection of proinsulin biosynthesis against streptozotocin toxicity which is comparable to control (non-streptozotocin exposed islets).

Effect of Agaricus bisporus PHA-B on proinsulin biosynthesis

The results of the incorporation of $^{14}$C-leucine into acid ethanol extractable proteins (proinsulin) in the presence of 17.5 μg/ml A. bisporus PHA-B, are presented in Table 6. From the results it would appear that there is no significant change in the incorporation of $^{14}$C-leucine into islet proteins in the
Fig. 25 - Nicotinamide protection against SZ inhibitory action. Preincubation was followed by exposure of SZ (1.5 mg) per 60-70 islets in the presence of different concentrations of nicotinamide (1-4 mg). Each result is expressed as the mean of % protection for six different experiments.
Table 6 - Effect of *A. bisporus* PHA-B (17.5 μg/ml) on pro-insulin biosynthesis, as studied by ¹⁴C-leucine incorporation into acid ethanol extract (proinsulin).

<table>
<thead>
<tr>
<th>A. bisporus PHA-B /μg/ml</th>
<th>Soluble fractions (proinsulin) cpm/mg/islet protein</th>
<th>%Proinsulin biosynthesis</th>
<th>Non-soluble fractions (other proteins) cpm/mg/islet protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>114027 ± 24</td>
<td>100</td>
<td>43166 ± 280</td>
</tr>
<tr>
<td>17.5</td>
<td>101315 ± 40</td>
<td>89</td>
<td>20634 ± 42</td>
</tr>
<tr>
<td>17.5+1.5 mg(SZ)</td>
<td>24520 ± 41</td>
<td>21.5</td>
<td>41298 ± 312</td>
</tr>
</tbody>
</table>
presence of *A. bisporus* PHA-B when compared to the control where no lectin was added.

**SECRETION OF INSULIN FROM ISOLATED ISLETS OF LANGERHANS OF RAT IN RESPONSE TO CHOLECYSTOKININ (CCK-4) AND ITS ANALOGUES**

Cholecystokinin, a gastrointestinal hormone, has also been implicated to act as a neurotransmitter and influence insulin release from the islets. CCK-4, a tetrapeptide, is a known such initiator of insulin release. It has the amino acid sequence *Trp-Met-Asp-Phe-NH₂*. This and its two analogues have been prepared in this laboratory by replacing the first amino acid tryptophan by p-glutamate and proline and their insulin releasing potentialities were tested. The results have been presented.

*Trp-Met-Asp-Phe-NH₂*

The results of the effect of CCK-4 on insulin release from isolated islets of Langerhans are shown in Fig. 26. The insulin release at the CCK-4 concentration of 10⁻¹⁰M is 68 ± 8 μU/5 islets/hr as compared to the control (2 mM glucose alone) in which the insulin release is only 30 ± 5 μU/5 islets/hr. The results of the effect of different concentration of CCK-4 on insulin release are presented in Fig. 26. With 2 mM glucose alone in the medium the insulin release is about 30 ± 5 μU/5 islets/hr. When CCK-4 is added in the medium the insulin release increases linearly with the concentration of the
Fig. 26 - Secretion of insulin from islets of Langerhans in response to different concentrations of cholecystokinin-4 (CCK-4). Each result is expressed as the mean of the rate of insulin release in terms of \( \mu U/5 \) islets/hr ± S.D. for six different experiments.
tetrapeptide.

Glp-Met-Asp-Phe-NH$_2$

When tryptophan of CCK-4 was replaced by p-glutamate the
tetrapeptide formed is not that active. The results of the
effect of this tetrapeptide on insulin release from islets of
Langerhans are presented in Fig. 27. While CCK-4 stimulates
insulin release at the concentration of 10$^{-10}$M, the new tetra­
peptide having p-glutamate in place of tryptophan is active at
higher concentration of 10$^{-8}$M at which the insulin release from
islets of Langerhans is 58 ± 5 μU/5 islets/hr as compared to
30 ± 5 μU/5 islets/hr, when no tetrapeptide was added in the
medium.

Pro-Met-Asp-Phe-NH$_2$

When tryptophan of the CCK-4 was replaced by proline, the
tetrapeptide thus formed also loses the activity. The results
of the effect of this tetrapeptide on insulin release from islets
of Langerhans are presented in Fig. 28. It is active at concen­
tration not less than 10$^{-8}$M. The insulin release at 10$^{-8}$M is
71 ± 5 μU/5 islets/hr as compared to 82 ± 8 μU/5 islets/hr in
the case of CCK-4.
Fig. 27 - Effect of CCK-4 analogue (Glp-Met-Asp-Phe-NH₂) on insulin release from islets of Langerhans. Each result is expressed as the mean of the rate of insulin release in terms of μU/5 islets/hr ± S.D. for six different experiments.

□ = 2 mM glucose
☑ = CCK-4
Fig. 28 - Insulin release from islets of Langerhans mediated by CCK-4 analogue (Pro-Met-Asp-Phe-ethyl). Each result is expressed as the mean of the rate of insulin release in terms of μU/5 islets/hr ± S.D. for six different experiments.
DISCUSSION
In the presented studies the technique of affinity chromatography has been employed for the preparation of homogeneous lectin fractions of *Agaricus bisporus*. The need for the development of more efficient and simple method for the purification of the lectin was felt especially when one of its fractions PHA-B was found to be able to release insulin and glucagon from the islets of Langerhans (Ewart et al., 1975). This finding has added to its importance as a tool for studying the mechanism of insulin and glucagon release from the islet cells. Earlier method of purification of the two fractions PHA-A and PHA-B of this lectin yielded only partially purified products (Presant and Kornfeld, 1972). Moreover, the method was a bit tedious and consisted of DEAE cellulose chromatography, sephadex G-150 gel filtration, phosphocellulose chromatography, a second time phosphocellulose chromatography and second time sephadex G-150 gel filtration. The partially purified materials of *A. bisporus*
PHA-A and PHA-B thus obtained gave six bands and two bands respectively on polyacrylamide gel electrophoresis.

By employing Con A - sepharose affinity chromatography and phosphocellulose chromatography it has been possible to obtain PHA-A and PHA-B fractions from A. bisporus purified to homogeneity each giving a single band on polyacrylamide gel electrophoresis and revealing the glycoprotein nature of the lectin. Thus the two step method consists of isolation of homogeneous A. bisporus PHA-A and PHA-B using Con A - sepharose chromatography followed by phosphocellulose column chromatography.

The molecular weight of A. bisporus PHA-A and PHA-B as determined by its elution profile from sephadex G-100 was 64,000 and 58,000 respectively, which is more or less similar as reported by Presant and Kornfeld (1972). The only other lectin which has been shown to possess insulin and glucagon releasing property was isolated from meadow mushroom Agaricus campestris (Sage and Connott, 1969). The molecular weight of this lectin was also approximately the same as that of A. bisporus PHA-A reported here. However, while A. bisporus PHA preparation consists of two fractions PHA-A and PHA-B, the lectin from A. campestris is a single material. The two mushrooms being genetically and morphologically distinct (Singer, 1961) also differ in their carbohydrate contents. The carbohydrate content of A. campestris as reported by Sage and Connott (1969) is 4%
while the carbohydrate content of _A. bisporus_ has been found to be 2.5% in the present studies.

Con A-sepharose affinity chromatography has been extensively used for the purification of various glycoproteins and hemagglutinins (Allan _et al._, 1972; Cuatrecases and Tell, 1973; Nachman _et al._, 1973; Steinemann and Stryer, 1973; Ahmad _et al._, 1977). However, to the best of the author's knowledge, lectins from _A. bisporus_ has not been purified with this method before.

**IN VITRO STUDIES ON LECTINS (ACARICUS BISPORUS PHA-B) MEDIATED INSULIN RELEASE AND _^{45}Ca^{2+}_ UPTAKE BY ISLETS OF LANGERHANS**

The effect of partially purified fraction of _A. bisporus_ PHA-B on insulin and glucagon release has been demonstrated by Ewart _et al._ (1975), while the other fraction PHA-A has been shown to have no effect on the hormone secretion from islets of Langerhans. The studies were carried out with the _A. bisporus_ PHA-B preparation which exhibited two bands on polyacrylamide gel electrophoresis. Therefore, it was not ideally pure preparation for detailed studies on the release of hormones from the islets cell which is supposed to require specific interaction between lectins and endocrine secretory cells.

The present studies have been carried out with a homogeneous _A. bisporus_ PHA-B preparation, which exhibited only
one band on polyacrylamide gel electrophoresis. The data presented here confirm the findings of Ewart et al. (1975) on A. bisporus PHA-B mediated insulin release from the islets.

In the present studies it has been shown that the sub-stimulatory dose of glucose for insulin release when added to the medium causes optimal \( O_2 \) uptake which is not altered by raising the glucose concentration to 10 folds. Thus contrary to insulin release, the rate of \( O_2 \) uptake does not seem to change by increasing the extracellular glucose concentration. Islets of Langerhans do survive in the presence of glucose and perform several metabolic functions viz, oxygen consumption, glucose oxidation (Gunnarsson et al., 1974) and these metabolic functions are inhibited by diabetogenic agents. Also, the purified lectin which is known to stimulate insulin release when added to the medium containing 2 mM glucose does not have any effect on \( O_2 \) uptake. The failure of the lectin to stimulate \( O_2 \) uptake indicates that the islet cell metabolism is not affected by lectin islet interaction. This is in agreement with the suggestion that the lectin stimulated insulin release is not energy dependent (Ewart et al., 1975). In the currently accepted view, peptide secretory process demonstrate a uniform requirement for energy (Stormorker, 1969). The present finding may therefore suggest that the observed argumentation of insulin release by lectins is due to cell injury. However, exposure of A. bisporus PHA-B to islets does not lead either to structural
damage of the islets at light microscopic level or to the impairment of islets responsiveness to glucose stimulation as evident from the present studies on insulin release by *A. bisporus* PHA-B in the presence of 2 mM glucose.

Many types of mammalian cells undergo some alterations in their behaviour when interacted with certain lectins. Such effects of lectins are well described in blast transformation (Nowell, 1960), induction of the release reactions in platelets (Majerus and Brodie, 1972), induction of a number of insulin like effects in fat cells (Cuatrecasas and Tell, 1973; Czech and Lynn, 1973). Our observations that the purified *A. bisporus* PHA-B as obtained by the described method stimulates insulin release from islets of Langerhans when incubated with 2 mM glucose are in agreement with those of Ewart et al. (1975).

The stimulation of insulin release has been found to be lectin dose dependent, where maximum insulin release is observed at lectin concentration at about 60 µg/ml. These observations together with the continuous recording of oxygen uptake by islets in the presence of lectin further confirm the maintenance of cellular integrity upto at least two hours (data not shown). The insulin release in the presence of 2 mM glucose and 2 mM glucose + 17.5 µg/ml *A bisporus* PHA-B progressed as a linear function of time for a period of one hour, but the increase in response to lectin is 3 fold as compared to 2 mM glucose alone.
The stepwise process of insulin release from the B-cell is now well established and the role of microtubules in \( \beta \)-granule migration has been suggested (Malaisse et al., 1971; Berlin, 1972). The chain of events involved in insulin secretion has been described as recognition by the B-cells of changes in the extracellular concentration of glucose, transmission of the message to the intracellular sites, the movement of insulin granules towards cell membrane and fusion of granular membrane with the cell membrane and finally extrusion of the granules to the extracellular space by emiocytosis. Keeping in view the energy independent nature of lectin stimulated insulin release its effect on the microtubular microfilamentous mediated acceleration of granule migration can be ruled out, since microtubular activity is believed to be energy dependent (Goodman et al., 1970).

Glucose stimulated insulin release from islets has been shown to be inhibited by duterium oxide (Lacy et al., 1968; Malaisse et al., 1971). Ewart et al. (1975) have also shown that *A. bisporus* lectin mediated insulin release is also inhibited by duterium oxide. This clearly assigns, that the lectin binding is at islet cell surface, because duterium oxide is known to modify intramolecular forces with resulting stabilization of protein configuration (Calfin et al., 1959). It is conceivable, therefore, that lectin induces alterations in the structure or arrangement of specific membrane components of
B-cells may in some way facilitate the process of exocytosis of insulin.

\(^{45}\text{Ca}^{2+}\) uptake by islets of Langerhans

The in vitro studies on the uptake of \(^{45}\text{Ca}^{2+}\) by isolated islets of Langerhans and the effect of Agaricus bisporus lectin fraction PHA-B presented in this dissertation show that this lectin is able to enhance \(^{45}\text{Ca}^{2+}\) uptake by islets in the presence of 2 mM glucose. The \(^{45}\text{Ca}^{2+}\) uptake has been found to be lectin dose dependent. If these data are compared with those of insulin release presented in this dissertation, it would appear that there is a definite correlation between lectin stimulated \(^{45}\text{Ca}^{2+}\) uptake by islets of Langerhans and the corresponding insulin release.

Gangerman et al. (1980) have shown acetylcholine-potentiated glucose stimulated insulin release from ob/ob mouse islets. However, they have demonstrated that there was no effect of acetylcholine on glucose oxidation and on basal insulin secretion at glucose concentration of 3 mM or less, but fortified at higher concentration. Their data showed a discrepancy between the effect of acetylcholine on insulin release and that of \(^{45}\text{Ca}^{2+}\) uptake – acetylcholine stimulated \(^{45}\text{Ca}^{2+}\) uptake at 3 mM glucose concentration but not at 11 mM. Similar conclusions have been drawn by other workers too (Hellman et al., 1978; Flott et al., 1978). Contrary to the acetylcholine...
stimulated $^{45}\text{Ca}^{2+}$ uptake by islets, our studies have shown that the lectin PHA-B stimulates $^{45}\text{Ca}^{2+}$ uptake by islets. It also stimulates basal insulin release at glucose concentration of 2 mM in the medium. The $^{45}\text{Ca}^{2+}$ uptake in terms of cpm/5 islets increases about 30 fold with 50 $\mu$g/ml lectin as compared to the control which contain 2 mM glucose but no lectin. The corresponding insulin release increases from 33 $\mu$U/5 islets/hr to 165 $\mu$U/5 islets/hr thus showing an increase of about 5 fold with 50 $\mu$g/ml lectin.

The results presented here are in agreement with the general hypothesis that $\text{Ca}^{2+}$ plays a role in stimulus secretion coupling (Douglas, 1968). Malaisse and coworkers (1971) have also shown that $^{45}\text{Ca}^{2+}$ incorporation was enhanced by insulin secretagogues like D-glucose, D-mannose, L-Leucine, tolbutamide, uninfluenced by compounds lacking insulin releasing activity (3-O-methyl-D-glucose, L-glucose, D-Leucine, D-galactose) and counteracted by inhibitors of insulin release (D-mannoheptulose, diazoxide, L-adrenaline). The stimulatory effect of $\text{A. bisporus}$ PHA-B appears to be similar to the insulin secretagogue in enhancing the $^{45}\text{Ca}^{2+}$ uptake and insulin release by islets.

In the previous studies on $^{45}\text{Ca}^{2+}$ uptake by islets, the extracellular space was compensated by washing without lanthanum (Malaisse-Lagae and Malaisse, 1971) or by using radioactive mannitol as an extracellular space marker (Hellman et al., 1971). Both the techniques were not satisfactory with regards
to the possibility of estimating the intracellular contents of $^{45}\text{Ca}^{2+}$. The mannitol method, does not compensate for $^{45}\text{Ca}^{2+}$ bound to the surface of cell and on cellular tissue component, washing without lanthanum permits a fraction of intracellular $^{45}\text{Ca}^{2+}$ to escape before the islets radioactivity is measured. The present studies have been carried out by loading the islets with $^{45}\text{Ca}^{2+}$ and after washing with buffer containing lanthanum chloride to prevent the loss of intracellular $^{45}\text{Ca}^{2+}$ from leakage by blocking transmembrane fluxes of $^{45}\text{Ca}^{2+}$ of loaded islets as described by Hellman et al., (1976). Using lanthanum washing technique, in response to 2 mM glucose and A. bisporus lectin it has been found that $^{45}\text{Ca}^{2+}$ uptake was enhanced by islets associated with insulin release. These results are in agreement with those of Hellman et al. (1976, 1977), where they have shown that various modifiers of insulin release (D-glucose, D-mannose, L-Leucine, tolbutamide) enhance the lanthanum non-displaceable $^{45}\text{Ca}^{2+}$ uptake by isolated pancreatic islets.

The present studies also show that the effect of lectin on $^{45}\text{Ca}^{2+}$ uptake in the presence of 2 mM glucose persist as evident from the time course studies carried out on isolated islets. The elevated $^{45}\text{Ca}^{2+}$ uptake as compared to control persists for at least two hours of incubation.

A very strict correlation between islets Ca$^{2+}$ uptake and insulin release has been demonstrated in islets of Langerhans (Malaisse-Lagae and Malaisse, 1971; Malaisse, 1975). The
effects of glucose and lectin on insulin release and $^{45}\text{Ca}^{2+}$ uptake suggest that glucose and lectin stimulated islets had incorporated $^{45}\text{Ca}^{2+}$ into the cell with a particularly high affinity for the ion with simultaneous insulin release. One of the possibilities is that glucose and lectin could enhance the total inflow of $^{45}\text{Ca}^{2+}$ by altering the transporting properties of B-cells. The precise things are that probably glucose and lectin by their stimulatory effect on islets, may have created new Ca$^{2+}$ storing compartment in B-cell. Such change may also involve an increase in the number of intracellular Ca$^{2+}$ binding sites, an increase in the affinity of the pre-existing sites and a sequestering of Ca$^{2+}$ within the membranes. Histochemical studies at the ultrastructural level have revealed that glucose promotes the deposition of Ca$^{2+}$ near the insulin secretory granules and plasma membrane of the B-cells (Herman and Hales, 1973; Schafer and Kloppel, 1974). This deposition of Ca$^{2+}$ due to glucose may result for the release of insulin. Agaricus bisporus PHA-B triggering the same type of effect on islets, thus promoting $^{45}\text{Ca}^{2+}$ uptake, may be the cause for stimulating insulin release from islets of Langerhans.

The sluggish behaviour of the $^{45}\text{Ca}^{2+}$ release as demonstrated by Hellman et al. (1976) is in striking contrast to the rapidity with which the glucose stimulated insulin secretion is affected by changes in the extracellular $^{45}\text{Ca}^{2+}$ concentration. When the rat pancreas was perfused with about 17 mM
D-glucose in the absence of $^{45}$Ca$^{2+}$; and the sudden inclusion of 2.5 mM Ca$^{2+}$ in the perfusion medium caused a prompt secretory response that reached its maximum within a couple of minutes (Grodsky, 1972). Conversely, the renewed withdrawal of Ca$^{2+}$ from the medium resulted in a quick drop of the secretory rate to basal value within 2 minutes (Grodsky, 1972). Hellman et al. (1973) observed that the withdrawal of $^{45}$Ca$^{2+}$ from islets perfused with 17 mM glucose and 0.1 mM iodoacetate caused a 50% drop of secretory rate within seven minutes. The rapidity with which the glucose induced insulin release is inhibited on withdrawal of Ca$^{2+}$ suggests that the secretory responsiveness is also dependent on a fairly labile Ca$^{2+}$ pool that is perhaps located in the B-cell plasma membrane.

As evident from the studies presented here the $^{45}$Ca$^{2+}$ uptake and insulin release is effected by lectin (A. bisporus PHA-B). If insulin release is controlled by ionic events in or near the B-cell plasma membrane, the simplest conceivable way of action of the lectin would be to bind directly on the plasma membrane or traditional receptors. The data presented here on the stimulation of $^{45}$Ca$^{2+}$ uptake by lectin suggests that, possibly the lectin induces alterations in the structure and arrangements of specific membrane components may in some way facilitate the process of $^{45}$Ca$^{2+}$ uptake and the process of exocytosis with resulting acceleration of hormone release.
From the results presented in Fig. 18, it would appear that basal insulin secretion at 2 mM glucose does not alter with age of the rats. This is in agreement with the findings of Lipson et al. (1981), however, when lectin PHA-B was added to islets isolated from different age groups of rats, in the medium containing 2 mM glucose, the effect of age is quite significant. The islets from younger age group i.e. one month old rat show 1.5 fold higher insulin secretion as compared to the islets from 12 month old rats. The slower response in the case of islets from older rats occurs despite the fact that islets from older rats are larger and contain more B-cells and that each B-cell stores more insulin than B-cells from younger rats (Gold et al., 1976; Kitahara and Adelman, 1979; Reaven et al., 1979).

Likewise, the effects of 2 mM glucose and A. bisporus PHA-B on uptake of $^{45}\text{Ca}^{2+}$ by islets on different age groups of rats are presented in Fig. 19 and 20. Here too, the $^{45}\text{Ca}^{2+}$ uptake by islets does not alter with age, but when the A. bisporus PHA-B was added to the medium, the $^{45}\text{Ca}^{2+}$ uptake by islets from one month old rats show more than 3 fold stimulation as compared to the islets isolated from 12 month old rats. When the incubation period was increased from 1 hour to 2 hours
the corresponding $^{45}$Ca$^{2+}$ uptake also increases showing the same pattern in different age groups. The slower response of islets isolated from older rats to glucose or leucine as compared to the response of islets from younger rats to the above sugar and amino acid with respect to insulin release has been reported by Reaven et al. (1979), Reaven and Reaven (1980).

It has been shown by Lipson et al. (1981) that decrease in the insulin release response of islets from older rats is associated with decreased adenylcyclase activity without alterations in phosphodiesterase and basal protein kinase activity. They have concluded that enzyme changes may play role in the diminished insulin release response to glucose from aging rats. The influence of adenylcyclase-cAMP system on the glucose stimulated insulin release in adult islets has been reported by Grill et al. (1974). Lipson et al. (1981) have also suggested that the glucose stimulated insulin release shows a diminished rate in aging rats whereas the basal insulin secretion remains unchanged. The results presented in this dissertation that there is a diminished rate of insulin release in aging rats in response to A. bisporus PHA-B, and the basal insulin secretion remains unchanged are in agreement with those of Lipson et al. (1981).

The low effectiveness of glucose in fetus compared with that on the adult might be caused by an inadequate intracellular concentration of cAMP as has been suggested by several workers.
(Lambert et al., 1970; Burr et al., 1971; Kervran et al., 1977).

On the other hand, no difference in cAMP concentration and in phosphodiesterase activity were observed in the pancreatic islets of 21 day old rat fetuses and their mothers (Mintz et al., 1973). Kervran and Randon (1980) have shown that theophylline potentiates the total insulin release elicited by glucose from 18.5 to 21.5 day of the gestation by an average of 65%. Similar findings have been also reported by other workers in adult rats (Brisson et al., 1972; Grill and Cerasi, 1974; Henquin, 1978). These findings suggest that insulin release by the islets through quantitative changes in the adenylcyclase - cAMP system is unlikely. It has been shown that in the case A. bisporus PHA-B, the potentiation of insulin release in the presence of substimulatory glucose concentration (2 mM) is independent of sources of metabolic energy. It does not alter islet glucose oxidation to CO₂, as shown in this dissertation, and incorporation of ³H-leucine into islet proteins (Ewart et al., 1975). Therefore its effect through the activation of adenylcyclase can be ruled out since this process would require energy for the release of insulin from B-cells. The stimulation of insulin release by PHA-B is dose dependent as has been shown in this dissertation and the stimulation requires only substimulatory glucose concentration. This also support the view that A. bisporus PHA-B stimulation of insulin release is independent of energy requirement.
In Fig. 15 and 16, it has been shown that \textit{A. bisporus} PHA-B potentiates the substimulatory glucose initiation of insulin release as well as $^{45}\text{Ca}^{2+}$ uptake by isolated islets of Langerhans. Although the requirement of extracellular Ca$^{2+}$ for stimulation of insulin release by physiological agents has long been established (Grodsky and Bennett, 1966; Milner and Hales, 1967; Curry \textit{et al.}, 1968). The rise in the concentration of cytosolic free Ca$^{2+}$ is the common link in the stimulus-secretion coupling for various secretory cells (Douglas, 1968; Rasmussen, 1972).

The relative importance of different possible sources of activator, Ca$^{2+}$ is still a matter of debate and there is no general agreement about the primary effect of glucose on the Ca$^{2+}$ movements that could bring about increase in cytosolic Ca$^{2+}$ which is required for the stimulation of insulin release by glucose and other potentiators (Ashwooth, 1971; Malaisse-Lagae, \textit{et al.}, 1971; Gerich \textit{et al.}, 1974). L-Leucine and L-arginine both increases the rate of retention of $^{45}\text{Ca}^{2+}$ by islets (Tixier-Vidal \textit{et al.}, 1976; Iatz-Buctier, 1977). Recently it has been shown that glucose stimulation results in an increase of $^{45}\text{Ca}^{2+}$ uptake without enhancing the total Ca$^{2+}$ content of the islet (Walters \textit{et al.}, 1982). It has been suggested that the enhanced uptake of $^{45}\text{Ca}^{2+}$ may be the consequence of increased permeability of cell membrane and increased cytosolic Ca$^{2+}$ ion as a result of mobilization of Ca$^{2+}$ from intracellular stores.
The *A. bisporus* PHA-B stimulated $^{45}$Ca$^{2+}$ uptake by islets as shown in the present studies is lectin dose as well as time dependent and there is a definite increase in the total $^{45}$Ca$^{2+}$ content of the islets in response to the lectin. The *A. bisporus* PHA-B mediated $^{45}$Ca$^{2+}$ uptake does not seem to mobilize $^{45}$Ca$^{2+}$ from intracellular stores of the B-cells. If the results of *A. bisporus* PHA-B mediated insulin release and $^{45}$Ca$^{2+}$ uptake by islets in different age groups of rats are compared, it would appear that $^{45}$Ca$^{2+}$ uptake is probably a pre-requisite for stimulatory action of *A. bisporus* PHA-B to release insulin.

The glucose stimulated uptake of $^{45}$Ca$^{2+}$ by islets of Langerhans is known to be energy dependent and substances that interfere with the energy production of islets viz, 2,4-dinitrophenol and antimycin A, causes loss of $^{45}$Ca$^{2+}$ uptake by the islets in response to a high glucose concentration (Hellman, 1979). In the case of *A. bisporus* PHA-B, as has been mentioned earlier, the stimulation of insulin release is not energy dependent (Swart et al., 1975). However, the present finding that *A. bisporus* PHA-B stimulates $^{45}$Ca$^{2+}$ uptake require further studies on energy dependent of the *A. bisporus* PHA-B stimulated $^{45}$Ca$^{2+}$ uptake by the islets of Langerhans. The effect of *A. bisporus* PHA-B on adenylcyclase-cAMP system has not been studied presently. The studies would further throw some light on the mechanism of *A. bisporus* PHA-B mediated insulin release.
Although its etiology is not fully known, maturity onset diabetes mellitus is a common disease in human population and may be caused due to altered insulin secretion and biosynthesis, increased adiposity, altered sensitivity to insulin and altered insulin action in addition to possibly dietary and exercise changes (Andres and Tobin, 1977; Davidson, 1979). Clinical human studies and in vivo animals studies have revealed, decreased unchanged or increased glucose stimulated insulin release in aging (Crockford, 1966; Dudl and Ensink, 1977; Andres and Tobin, 1977; Davidson, 1979; Yoshino et al., 1979; Soejodibroto et al., 1979). In vitro studies have demonstrated significantly diminished glucose stimulated insulin release from islets of aging rats (Gold et al., 1976; Kitahara and Adelman, 1979; Reaven et al., 1979; Lipson et al., 1981). The results of the effect of A. bisporus PHA-B on insulin release from islets of aging rats also show a diminished rate of insulin secretion, hence, are in agreement with those mentioned above. The effect of lectin on insulin release and $^{45}$Ca$^{2+}$ uptake by islets of aging rats is the first report presented in this dissertation.

If the concept of specific interaction of A. bisporus PHA-B and its receptors leading to some conformational changes in the structure of the membrane of the B-cells is accepted, then it would be conceivable that aging has some effect on the structural or conformational integrity of the B-cell membrane which would lead to alterations in the responsiveness
of B-cell receptors to *A. bisporus* PHA-B binding which in turn would effect $^{45}$Ca$^{2+}$ uptake by islets and consequently exocytosis of insulin. However, more work is required to understand the exact mechanism of *A. bisporus*, lectin mediated insulin release.

**EFFECT OF STREPTOZOTOCIN-NICOTINAMIDE INTERACTION ON PRO-INSULIN BIOSYNTHESIS IN ISOLATED RAT ISLETS OF LANGERHANS: EFFECT OF LECTINS THEREON**

The present studies demonstrate that streptozotocin exerts a direct and immediate inhibitory effect on $^{14}$C-leucine incorporation into isolated islets of Langerhans. Many workers have shown that glucose stimulates the rate of incorporation of exogenous amino acids into proinsulin (Lin et al., 1972; Permutt and Kipnis, 1972). This stimulatory effect of glucose has been postulated as due to enhanced formation of RNA (Puchinger and Wacker, 1972). It is known that glucose stimulates the synthesis of proinsulin in preference to other islet proteins (Morris and Korner, 1970; Bansal et al., 1980).

Almost complete protection against streptozotocin toxicity to the B-cell with respect to insulin release has been demonstrated when nicotinamide was injected simultaneously to rat or up to two hours of injection of streptozotocin (Dulin and Wyse, 1969; Stauffacher et al., 1970). Exposure of isolated rat
islets of Langerhans to streptozotocin has been shown to inhibit subsequent glucose induced insulin release, whereas the presence of nicotinamide during streptozotocin exposure protects the insulin release (Golden et al., 1971). Other metabolic functions of islets cells like glucose oxidation are also reversed by nicotinamide, when exposed to streptozotocin (Wyse and Dulin, 1971; Gunnarsson et al., 1974).

Streptozotocin when incubated alone with islets produces 79% inhibition of the incorporation of the tracer into acid ethanol extractable proteins which predominantly consists of proinsulin while nicotinamide when incubated simultaneously with streptozotocin protects the proinsulin biosynthesis. The protection of nicotinamide is dose dependent and the complete protection is brought about by 4 mg nicotinamide against streptozotocin concentration of 1.5 mg per 60-70 islets in the incubation medium. Keeping in mind the streptozotocin specificity for B-cells and the proinsulin as a predominant protein in the acid-ethanol extraction procedure, the incorporation of the tracer into other non-insulin proteins of the islets can be neglected for the present studies. When lectin (A. bisporus PHA-B) was added in the medium along with streptozotocin no significant change was observed in the incorporation of the labelled amino acid into the acid-ethanol extractable protein, that consists of predominantly insulin/proinsulin. A. bisporus PHA-B which probably binds with the receptor on B-cell membrane
causing conformational changes resulting in the secretion of insulin does not affect the toxic action of streptozotocin, probably due to their different sites of interaction on the B-cell membrane.

It has been demonstrated that streptozotocin can produce DNA damage (Bhuyan et al., 1976) and suggested that the repair of DNA by poly (ADP-ribose) polymerase which utilizes NAD$^+$ (Smulson et al., 1977) results in the reduction of NAD$^+$ (Ho and Hashim, 1967; Slonim et al., 1976). Streptozotocin, depresses NAD$^+$, thus inhibiting glucose induced insulin release, whereas nicotinamide prevents it (Golden et al., 1971). Streptozotocin which produces DNA damage, also depresses the NAD$^+$. Nicotinamide, a precursor of NAD$^+$ fulfils this deficit, causing the protection against streptozotocin inhibitory action. The pro-insulin biosynthesis as shown by the incorporation of $^{14}$C-leucine into proinsulin in the present study may also be similarly affected by streptozotocin and the protection of the same by increasing doses of nicotinamide may be preventing the decrease of NAD$^+$, thus maintaining the level of poly (ADP-ribose) polymerase which protects DNA from damage.

Recently, Bennett and Pegg (1981) have demonstrated that streptozotocin is a potent methylating agent reacting with DNA in vitro to form methylated purines. It produces significant methylation of pancreatic DNA which, if concentrated in the B-cell may account for their destruction. Pretreatment with
nicotinamide reduces the extent of methylation of pancreatic DNA.

Our observations that nicotinamide can reverse the streptozotocin inhibited insulin/proinsulin biosynthesis on the B-cell is in agreement with the above findings. Nicotinamide by protecting the DNA against alkylation, by streptozotocin, probably helps in the biosynthesis of proinsulin/insulin in B-cell, which proceeds undisturbed.

SECRETION OF INSULIN FROM ISOLATED ISLETS OF LANGERHANS OF RAT IN RESPONSE TO CHOLECYSTOKININ (CCK-4) AND ITS ANALOGUES

The question why oral administration of glucose results in a considerably great rise of insulin secretion than its i.v. administration at the same rate was partially answered when several gastrointestinal factors were shown to evoke insulin secretion. Among the gastrointestinal polypeptides suggested to possess insulinotropic activity are cholecystokinins (CCKs), gastric inhibitory polypeptide (GIP), secretin, gastrin, vaso-active intestinal polypeptide (VIP) and the gut peptide.

The insulinotropic role of CCK is not yet clear. Due to difficulties in measuring CCK, little information is available regarding its concentration in circulating blood and its release by specific nutrients. Also, cholecystokinin, exists in several molecular forms, viz. CCK-4, CCK-8, CCK-33 and CCK-39
(Mutt, 1978), whose effects on insulin release are diverse depending upon the animal species (Robinovitch and Dupre, 1974; Rehfeld et al., 1980; Ahren and Lundquist, 1982; Szecowka et al., 1982).

In the present studies CCK-4 (Trp-Met-Asp-Phe-NH₂) and its two analogues Glp-Met-Asp-Phe-NH₂ and Pro-Met-Asp-Phe-NH₂, synthesized in this laboratory, have been tested for their insulinotropic effect on isolated rat islets of Langerhans. The CCK-4 stimulates insulin release from isolated islets at a concentration of 10⁻¹⁰M. The insulin release mediated by CCK-4 is dose dependent and increases with the concentration of peptide. These results are in agreement with those of Rehfeld, 1971; Rehfeld et al., 1980; where CCK-4 has an immediate and potent effect on islet cell secretion. It has been suggested by Rehfeld et al. (1971) that human islets may also have receptors for CCK-4, and thus it may act as a neurotransmitter or neurohormone in human pancreas. The potent effect of CCK-4 on insulin secretion and B-cell growth (Larsson et al., 1977, 1968) is of considerable importance and thus a suitable analogue of CCK-4 may be of value in the treatment of diabetes mellitus.

When Trp was replaced by Glp and Pro, the potency of the CCK-4 is considerably reduced. The insulin release from islets is observed at 10⁻⁸M peptide concentration as compared to the CCK-4 (10⁻¹⁰M). Thus by replacing Trp the potency of the CCK-4 is reduced. It may be suggested that Trp is necessary for the
expression of insulin releasing activity of CCK-4. This is in agreement with Rehfeld et al. (1980), where they have shown that removal of Trp from NH₂-terminal, extension of glycine, greatly reduces the secretagogue activity. However, the effect of these analogues have not been tested on other animal species as far. It will be of considerable interest to test the insulinotropic activity of several such analogues on different animal species, since it is known that the different molecular form of CCK are specific for their activity (Szecowka et al., 1982).
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SUMMARY AND CONCLUSION
The endocrine cells have a unique property of being able to respond to a specific physiological stimulus with a resultant release of stored hormone in a modulated fashion. The mechanism through which extracellular factors govern the intracellular events in the endocrine pancreas remain poorly defined. The secretion of insulin by pancreatic B-cells is stimulated by sugars, amino acids, ketone bodies, hormones, cAMP and its derivatives. Insulin release is the result of a series of phenomenon including synthesis of proinsulin in the endoplasmic reticulum, transport of proinsulin to the Golgi apparatus, formation and maturation of granules, conversion of proinsulin to insulin, transfer or conversion of granules to a labile form and finally release of insulin from the B-cells. Toxins from *Agaricus bisporus* and *Agaricus campestris* stimulate insulin release from islets of Langerhans. Binding of *A. bisporus* PHA-B stimulated insulin release were inhibited in parallel by a glycopeptide containing the oligosaccharide receptor for lectin, thus lectin binding appears to be essential for the expression
of its insulin releasing activity. The studies have been carried out with the view to obtain some information regarding the effect of some extracellular events on the mechanism of insulin release. Lectin PHA-B and streptozotocin have been used for our studies on their interaction with their possible receptors on B-cells membranes.

ISOIATION, PURIFICATION, AND CHARACTERIZATION OF LECTINS FROM AGARICUS BISPORUS

The need for the development of a more efficient and simple method for the purification of Agaricus bisporus lectin was felt especially when one of its fraction PHA-B was found to be able to release insulin from the islets of Langerhans.

The present purification procedure consists of ConA-sepharose affinity chromatography and phosphocellulose chromatography. A. bisporus PHA was purified with a recovery of 90.8% and 6 fold purification by ConA-sepharose column chromatography when crude extract (NaCl extract and heat treated) was passed through ConA-sepharose column and was eluted with 0.1 M mannose in phosphate buffered saline, 0.02 M, pH 7.2. The preparation was resolved into two hemagglutinating fractions by phosphocellulose column chromatography thus about 80% of the hemagglutinating activity of the ConA-sepharose
eluate was eluted at pH 5.85 (fraction PHA-A), whereas 15% of the hemagglutinating activity was released from the column when the pH of the eluting buffer was raised to 8.2.

The Con A-sepharose eluate exhibited one major and one minor band on polyacrylamide gel electrophoresis corresponding to PHA-A and PHA-B. The PHA-A and PHA-B obtained by phosphocellulose column chromatography exhibited single band separately on polyacrylamide gel electrophoresis, showing homogeneous preparation of the two PHA's fractions.

The molecular weights of the purified A. bisporus PHA-A and PHA-B as determined by sephadex G-100 gel filtration were 64,100 and 58,000 respectively. The neutral sugars content of the purified PHA was found to be 2.5% as estimated by phenol sulphuric acid method.

EFFECT OF AGARICUS BISPORUS PHA-B ON GLUCOSE METABOLISM IN ISOLATED ISLETS OF LANGERHANS

These studies were carried out in order to study the effect of A. bisporus PHA-B on oxygen uptake by the isolated islets of Langerhans. It was found that in the presence of 2 mM glucose alone and 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B (that produces half maximal stimulation of insulin release) there was no significant difference in the μl of O₂ uptake.
Similarly no significant change in O$_2$ uptake was observed when glucose concentration was raised to 20 mM and keeping PHA-B concentration 17.5 $\mu$g/ml. Thus it appeared that the A. bisporus PHA-B did not alter the rate of islet glucose metabolism.

IN VITRO STUDIES ON AGARICUS BISPORUS PHA-B MEDIATED INSULIN RELEASE FROM ISLETS OF LANGERHANS

The study was carried out in order to determine the insulin secreting property of the purified A. bisporus PHA-B. For in vitro studies islets were incubated with A. bisporus PHA-B (17.5 $\mu$g-100 $\mu$g/ml) in the presence of 2 mM glucose in KRB medium. Samples were drawn at an interval of one hour and the insulin content was estimated by radioimmunoassay.

It was found that at 2 mM glucose + 17.5 $\mu$g/ml PHA-B the insulin release was 109 $\pm$ 5 $\mu$U/5 islets/hr as compared to the control (2 mM glucose), which was 33 $\pm$ 5 $\mu$U/5 islets/hr. Thus, there was 3.3 fold increase in insulin release in response to the lectin as compared to the control. The stimulation of the insulin release was found to be lectin dose as well as time dependent. The maximal stimulation of the insulin release was obtained at lectin concentration of about 60 $\mu$g/ml after which the release of insulin tends to be stationary. The lectins mediated insulin release from islets of Langerhans proceeded as a linear function of time for about one hour.
The insulin release in presence of 2 mM glucose alone and 2 mM glucose + 17.5 μg/ml (A. bisporus PHA-B) was enhanced from the beginning and after 20 minutes of incubation the release in the presence of A. bisporus PHA-B was about three times as compared to 2 mM glucose alone and the same proportion was maintained throughout the one hour period of incubation.

It is conceivable, therefore, that lectin induces alterations in the structure or arrangement of specific membrane components may in some way facilitate the process of exocytosis with resulting acceleration of insulin release.

45Ca uptake studies in the presence of glucose and A. bisporus PHA-B by isolated islets of langerhans

D-glucose alters the state of Ca2+ in islets of Langerhans and enhances the rate of uptake of isotope. It has been assumed that B-cells are responsible for observed stimulatory effect on islet calcium uptake and a very strict correlation between islet radioactivity and insulin release has been demonstrated. These studies were carried out in order to find out the ability of the purified A. bisporus to enhance 45Ca2+ uptake by islets as A. bisporus PHA-B is also an insulin secretagogue.

It was found that in the presence of 2 mM glucose + 17.5 μg/ml A. bisporus PHA-B the 45Ca2+ uptake by islets was enhanced more than eleven folds as compared to the control.
(2 mM glucose) whereas when the lectin concentration was raised to 50 μg/ml, the corresponding \(^{45}\text{Ca}^{2+}\) uptake by islets was increased more than 30 folds as compared to the control. The \(^{45}\text{Ca}^{2+}\) uptake was found to be lectin dose and time dependent. The rate of the \(^{45}\text{Ca}^{2+}\) uptake increased with the concentration of the lectin and the maximal uptake was achieved at lectin concentration of about 60 μg/ml and then the rate slows down until it reached a stationary phase at about 75 μg/ml lectin concentration. Thus there was a strict correlation between insulin release and \(^{45}\text{Ca}^{2+}\) uptake by islets. The maximal insulin release as well as maximal \(^{45}\text{Ca}^{2+}\) uptake was achieved at lectin concentration of about 60 μg/ml. Both the studies were carried out in the same set of experiment.

For time course studies islets were incubated with 2 mM glucose, 2 mM glucose + 17.5 μg/ml \textit{A. bisporus} PHA-B and \(^{45}\text{Ca}^{2+}\) for various periods of time. The rate of \(^{45}\text{Ca}^{2+}\) uptake increased linearly with time for about 120 minutes after which it became stationary. There was a significant stimulation in the uptake of \(^{45}\text{Ca}^{2+}\) after the addition of \textit{A. bisporus} PHA-B in the medium (17.5 μg/ml) and thus at this lectin concentration the stimulation of \(^{45}\text{Ca}^{2+}\) uptake is more than 12.5 folds as compared to the control (2 mM glucose).

It may be concluded that \textit{Ca}^{2+} plays a vital role in stimulus secretion coupling and the insulin release and \(^{45}\text{Ca}^{2+}\) uptake is affected by the lectin (\textit{A. bisporus} PHA-B). If insulin release
is controlled by ionic events in or near the B-cell membrane
the action of the lectin would be to bind directly to the plasma
membrane or the traditional receptors. The stimulation of
\(^{45}\text{Ca}^{2+}\) uptake by lectin also suggests that the lectin induces
alterations in the structure and arrangement of specific membrane
component may in some way facilitate the process of \(^{45}\text{Ca}^{2+}\) up-
take and the process of exocytosis of hormone release.

INSULIN RELEASE IN AGING IN RESPONSE TO AGARICUS BISPORUS
PHA-B FROM ISLETS OF LANGERHANS

Maturity onset diabetes mellitus may be caused due to
altered insulin secretion and biosynthesis, increased adiposity
and altered insulin action. It has been demonstrated that
insulin mediated insulin release is diminished in aging.

For \textit{in vitro} studies of the effect of \textit{A. bisporus} PHA-B
on insulin release and \(^{45}\text{Ca}^{2+}\) uptake by the islets isolated from
1, 3, 6 and 12 month old rats were incubated with 2 mM glucose,
2 mM glucose + 17.5 \(\mu\text{g/ml} \text{ A. bisporus PHA-B}\) and \(^{45}\text{Ca}^{2+}\). It
was found that the basal insulin release in the presence of
2 mM glucose was almost the same in all the group of islets
irrespective of the age of the rats.

The \textit{A. bisporus} PHA-B stimulated insulin release quite
significantly in younger animals. The increase in the release
of insulin in the presence of lectin in the case of islets
isolated from one month old rat was 6 folds as compared to the control (2 mM glucose alone) whereas the increase in the insulin release in the presence of the lectin was 2.4 folds as compared to the control in the case of islets isolated from 12 month old rat. There was also a 1.5 fold increase in insulin release in the case of islets of 1 month old rat as compared to the islets of 12 month old rat in response to lectin (A. bisporus PHA-B).

It may be concluded that insulin release in aging in response to A. bisporus PHA-B follows the same pattern as the glucose stimulated insulin release and if the concept of specific interaction of A. bisporus PHA-B and receptors leading to conformational changes in the structure of the membrane of B-cells is accepted, then it would be conceivable that aging has some effect on the structural or conformational integrity of B-cell membrane which would lead to alteration in the responsiveness of B-cell to A. bisporus PHA-B.

$^{45}$Ca$^{2+}$ UPTAKE BY ISLETS OF DIFFERENT AGE GROUPS IN PRESENCE OF AGARICUS BISPORUS PHA-B

The corresponding $^{45}$Ca$^{2+}$ uptake studies were carried out by islets isolated from 1, 3, 6 and 12 month old rats in response to 2 mM glucose and A. bisporus PHA-B. It was found that like insulin secretion the $^{45}$Ca$^{2+}$ uptake by islets in response to 2 mM glucose did not alter with age and when the incubation
period was increased from 1 hour to 2 hour there was no change observed in the uptake.

The stimulation of \(^{45}\text{Ca}^{2+}\) uptake by islets in response to \(A.\ bisporus\ \text{PHA}-B\) (17.5 \(\mu\text{g/ml}\)) was maximum in the islets isolated from younger animals as compared to the older ones. The uptake higher in response to lectin was 54.5 fold in the case of islets isolated from 1 month old rat as compared to the control (2 mM glucose) whereas the increase in \(^{45}\text{Ca}^{2+}\) uptake was 20.4 fold in the case of islets of 12 month old rat as compared to the control. There was an increase in lectin stimulation of \(^{45}\text{Ca}^{2+}\) uptake in the case of islets of 1 month old rat by 3 fold as compared to the islets which were isolated from 12 month old rat.

The conclusion may be drawn that \(^{45}\text{Ca}^{2+}\) uptake is probably a prerequisite for the stimulatory action of \(A.\ bisporus\ \text{PHA}-B\) for insulin secretion and the interaction of PHA-B and its receptor which leads to conformational changes in the B-cell membrane would affect \(^{45}\text{Ca}^{2+}\) uptake and consequently exocytosis of insulin. However, more work is required to understand the exact mechanism of \(A.\ bisporus\ \text{PHA}-B\) mediated insulin release.

**EFFECT OF STREPTOZOTOCIN-NICOTINAMIDE INTERACTION ON PROINSULIN BIOSYNTHESIS IN ISOLATED ISLETS OF LANGERHANS: EFFECT OF LECTINS THEREON**

Streptozotocin is a known diabetogenic agent whose toxic
action can be reversed by nicotinamide. In order to study the effect of streptozotocin-nicotinamide interaction these studies were performed by incorporation of $^{14}$C-leucine into acid-ethanol extractable proteins (proinsulin/insulin).

It was found that by increasing the concentration of streptozotocin from 0.05 to 1.5 mg per 60-70 islets, the biosynthesis of total islet protein decreased correspondingly as compared to the control (non-streptozotocin exposed islets) and at streptozotocin concentration of 1.5 mg per 60-70 islets the total protein biosynthesis was retarded by 5.5 fold as compared to the control. By increasing the concentration of nicotinamide from 0.5-4 mg per 60-70 islets, keeping streptozotocin concentration constant (1.5 mg), the inhibitory action of streptozotocin was reversed and at nicotinamide concentration of 4 mg in the medium the protein biosynthesis was restored to control value.

Similarly, the addition of streptozotocin (0.05-1.5 mg/60-70 islets) in the medium resulted in the inhibition of $^{14}$C-leucine incorporation into acid-ethanol extractable proteins (proinsulin). It was found that at streptozotocin concentration 1.5 mg/60-70 islets, there was 79% inhibition of the incorporation of the tracer into proinsulin and thus the proinsulin biosynthesis was retarded to 21% as compared to the control.

For protective studies nicotinamide (0.5-4 mg/60-70 islets) was incubated at constant concentration of streptozotocin
(1.5 mg). It was observed that at nicotinamide concentration 4 mg/60-70 islets, 100% protection was achieved against the streptozotocin toxicity.

There was no significant change in the incorporation of $^{14}$C-leucine into islet proteins and proinsulin in the presence of A. bisporus PHA-B. There was a 11% decrease in the incorporation radioactivity in acid ethanol soluble protein as compared to the control. The decreased radioactivity counts in the acid-ethanol extracts of islets may be due to the release of stored hormone in the B-cells.

It may be concluded that streptozotocin a diabetogenic agent, inhibits the proinsulin biosynthesis by depressing NAD$^+$ which is required by poly (ADP-ribose) polymerase for the repair of DNA, the damage caused by streptozotocin. Nicotinamide a precursor of NAD$^+$ may be preventing the decrease of NAD$^+$, thus maintaining the level of poly (ADP-ribose) polymerase which protects DNA from damage thus protecting proinsulin biosynthesis. Alkylation of DNA bases in the pancreas by streptozotocin may be another explanation for the inhibition of proinsulin biosynthesis, whereas nicotinamide reduces the extent of methylation and thus preventing proinsulin biosynthesis. A. bisporus PHA-B does not exert any effect on the toxic action of streptozotocin, probably due to their different sites of interaction on B-cell membrane.
SECRETION OF INSULIN FROM ISOLATED ISLETS OF LANGERHANS
IN RESPONSE TO CHOLECYSTOKININ (CCK-4) AND ITS ANALOGUES

Cholecystokinin is located in central and peripheral nerves and their nerve terminals have been found in pancreatic islets which have known insulinotropic property. Among CCKs, CCK-4 (Trp-Met-Asp-Phe-NH₂) is the most effective one. CCK-4 and its two analogues Glp-Met-Asp-Phe-NH₂ and Pro-Met-Asp-Phe-NH₂ were synthesized in this laboratory and were studied with respect to insulin releasing property.

The insulin release from the islets of Langerhans at the CCK-4 (Trp-Met-Asp-Phe-NH₂) concentration of 10⁻¹⁰ M was 68 ± 8 μU/5 islets/hr as compared to the control (2 mM glucose) which was 30 ± 5 μU/5 islets/hr. The insulin release increased linearly with the concentration of the tetrapeptide. When tryptophan of the CCK-4 was replaced by p-glutamate the tetrapeptide formed (Glp-Met-Asp-Phe-NH₂) was not that potent. This tetrapeptide was active at higher concentration of 10⁻⁸ M. Similarly when tryptophan of the CCK-4 was replaced by proline the tetrapeptide thus formed (Pro-Met-Asp-Phe-NH₂) also lost the activity. It was active at concentration not less than 10⁻⁸ M.
It may be suggested that tryptophan is necessary for the expression of insulin releasing activity of the CCK-4. Any substitution on N-terminal may be the cause of less activity as shown by the present studies. However, it will be of considerable interest to test the insulinotropic activity of these analogues on other animal species as CCKs are species specific for their activity.