REVIEW OF LITERATURE
3. REVIEW OF LITERATURE

3.1. Newer Trends in the Insulin Formulation

3.1.1. Brief History of Insulin

Insulin was discovered in 1921 by Banting and Best who demonstrated the hypoglycemic action of an extract of pancreas prepared after the degradation of the exocrine part due to ligation of pancreatic duct. It was first obtained in crystalline form in 1926 and the chemical structure was fully worked out in 1956 by Sanger.

Insulin is a two-chain polypeptide having 51 amino acids and molecular weight of about 6000. The A chain has 21 amino acids, while B chain has 30 amino acids as shown in Fig-1. There are minor differences between human, porcine and bovine insulin as shown in Table 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>A-Chain</th>
<th>B-Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8th Amino acid</td>
<td>10th Amino acid</td>
</tr>
<tr>
<td>Human</td>
<td>THR</td>
<td>ILEU</td>
</tr>
<tr>
<td>Pork</td>
<td>THR</td>
<td>ILEU</td>
</tr>
<tr>
<td>Beef</td>
<td>ALA</td>
<td>VAL</td>
</tr>
</tbody>
</table>

THR- Threonine; ALA-Alanine; ILEU: Isoleucine; VAL: Valine

Insulin is synthesized in the ß cells of pancreatic islets as a single chain peptide preproinsulin (110 amino acids) from which 24 amino acids are first removed to produce pro-insulin (Fig-2). The connecting or ‘C’ peptide (35 amino acids) is split off by proteolysis in golgi apparatus and both are stored in granules within the cell. The ‘C’ peptide is secreted in the blood along with the insulin.
Fig 1: Human insulin; Insulin molecule with two chains joined by two disulfide bridges

Fig 2: Human Proinsulin; simplified structure depicting the main features. The connecting peptide, having 35 amino acids, is split off leaving insulin molecule with two chains joined by two disulfide bridges
3.1.2. Pharmacology and Physiology of Insulin and its associated Problems

Regulation of insulin secretion

Under basal condition approximately 1U insulin is secreted per hour by human pancreas. Much larger quantity is secreted after every meal. Secretion of insulin from $\beta$ cells is regulated by chemical, hormonal, and neural mechanisms.

Chemical: The $\beta$ cells have a glucose sensing mechanism dependent on entry of glucose into $\beta$ cells (through the aegis of a glucose transporter) and its phosphorylation by glucokinase. Activation of the glucoreceptor indirectly causes partial depolarization of the $\beta$ cells and increases intracellular $Ca^{2+}$ availability (due to increased influx, decreased efflux and release from intracellular stores) that leads to exocytotic release of insulin. Other nutrients that can evoke insulin release are- amino acids, fatty acids and ketone bodies, but glucose is the principal regulator.

Hormonal: A number of hormones, e.g. growth hormone, corticosteroids, thyroxine modify insulin release in response to glucose. Prostaglandin E (PGE) has shown to inhibit insulin release. More important are the intra-islet paracrine interactions between the hormones produced by different types of islet cells. The $\beta$ cells constitute the core of the islets and are the most abundant cell type. The $\alpha$ cells, comprising 25% of the islet cell mass, surround the core and secrete glucagon. The D cells (about 10%) elaborating somatostatin are interspersed between the $\alpha$ cells. There are some PP (or F) cells (pancreatic polypeptide containing) also. Somatostatin inhibits release of both insulin and glucagon. Glucagon evokes release of insulin as well as somatostatin. Insulin inhibits glucagon secretion. The three hormones released from closely situated cells influence each other's secretion and appear to provide fine-tuning of their output in response to metabolic needs.
**Fig 3: Regulation of insulin secretion**

*Neural:* The islets are richly supplied by sympathetic and vagal nerves.

- Adrenergic $\alpha_2$ receptor activation inhibits insulin release (predominant) by inhibiting $\beta$ cells adenyl cyclase.
- Adrenergic $\beta_2$ stimulation increases insulin release (less prominent) by stimulating $\beta$-cells adenyl cyclase.
- Cholinergic- muscarinic activation by Acetyl choline or vagal stimulation causes insulin secretion through IP$_3$/DAG-increased intracellular Ca$^{2+}$ in the $\beta$ cells.

These neural influences appear to govern both basal as well as evoked insulin secretion, because the respective blocking agents have effects opposite to that mentioned above. The primary central site of regulation of insulin secretion is in the hypothalamus: stimulation of ventrolateral nuclei evokes insulin release, whereas stimulation of ventromedial nuclei has the opposite effect.

*Physiological actions of insulin*

The actions of insulin and the results of its deficiency can be summarized as:
It facilitates glucose transport across cell membrane; skeletal muscle and fat are highly sensitive. The availability of glucose intracellularly is the limiting factor for its utilization in these and some other tissues. However, glucose entry in liver, brain, RBC, WBC, and renal medullary cells is largely independent of insulin. Ketoacidosis interferes with glucose utilization by brain leads to diabetic coma. Muscular activity induces glucose entry in muscle cells without the need for insulin. As such, exercise has insulin-sparing effect.

The first step in intracellular utilization of glucose is its phosphorylation to form glucose-6-phosphate. This is enhanced by insulin through increased production of glucokinase. Insulin facilitates glycogen synthesis from glucose in liver, muscle and fat by stimulating the enzyme glycogen synthetase. It also inhibits phosphorylase leads to decreased glycogenolysis in liver.

Insulin inhibits gluconeogenesis (from protein) in liver by gene mediated decreased synthesis of phosphoenol pyruvate carboxy kinase. In insulin deficiency, proteins and amino acids are funneled from peripheral tissues to liver where these are converted to carbohydrate and urea. Thus, in diabetes there is underutilization and over production of glucose leads hyperglycemia leads glycosuria.

Insulin inhibits lipolysis in adipose tissue and favors and triglyceride synthesis. In diabetes increased amount of fat is broken down due to unchecked action of lipolytic hormones (glucagon, Adr, thyroxine etc.) leads to increase in free fatty acids (FFA) in blood leads to taken up by liver to produce acetyl CoA. Normally acetyl CoA is resynthesized to fatty acids and triglycerides, but this process is reduced in diabetics in acetyl CoA is diverted to produce ketone bodies (Acetone, Acetoacetate, β-hydroxy-butyrate). The ketone bodies are released in blood-partly used up by muscle and heart as energy source, but when their capacity is exceeded, ketonemia and ketonuria result.
Insulin stimulates transcription of vascular endothelial lipoprotein lipase and thus increases clearance of VLDL and chylomicrons.

Insulin facilitates amino acids (AA) entry and their synthesis into proteins in muscle and possibly other cells. Insulin deficiency leads to protein breakdown and in turn AAs are released in blood and in turn taken by liver and converted to pyruvate, glucose and urea. The excess urea produced is excreted in urine resulting in negative nitrogen balance. Thus, catabolism takes the upper hand over anabolism in the diabetic state. Most of the above metabolic actions of insulin are excreted within seconds or min and are called the rapid actions. Others involving DNA mediated synthesis of glucose transporter and some enzymes of AA metabolism have a latency of few hours- the intermediate actions. In addition insulin exerts long-term effects on multiplication and differentiation of cells.

**Mechanism of action**

Insulin acts on specific receptors located on the cell membranes of practically all cells, but their density depends on the cell type: liver and fat cells are very rich. The insulin receptor has been isolated and found to be heterotetrameric glycoprotein consisting of 2 α and 2β subunits linked together by disulfide bonds. The α subunits carry insulin binding sites while the β subunits have tyrosine protein kinase activity.

Binding of insulin to α subunits induces aggregation and internalization of the receptor along with the bound insulin molecules. This activates tyrosine kinase activity of the β subunits leads to tyrosine residues of the β subunits get autophosphorylated so that the activity of this subunit to phosphorylate tyrosine residues of other substrate proteins increased. In turn a cascade of phosphorylation and dephosphorylation reactions is set into motion resulting in stimulation or inhibition of enzymes involved in the rapid metabolic actions of insulin. Certain second messengers like
phosphatidyl inositol glycan (PIG) and DAG, which are generated through activation of a specific phospholipase C are also believed to mediate the action of insulin on metabolic actions of insulin on metabolic enzymes.

**Fate of insulin**

Insulin is distributed only extracellularly. It is a peptide-degraded in the gastro intestinal tract (g.i.t) if given orally. Injected insulin or that released from pancreas is metabolized primarily in liver and to a smaller extent in kidney and muscles. Nearly half of the insulin entering portal vein from pancreas is inactivated in the first passage though liver. Thus, normally liver is exposed to much higher concentrations (4-8 folds) of insulin than are other tissues. As above degradation of insulin after receptor mediated internalization occurs to variable extent in most target cells. During biotransformation the disulfide bonds are reduced – A and B chains are separated. These are further broken down to the constituent amino acids. The plasma $t_{1/2}$ is 5-9 min.

**3.1.3. Discovery and Development of Insulin**

The discovery of insulin by Banting, Best, and Macleod is one of the miraculous achievements of twentieth century medicine (Banting et al., 1922; Banting & Best, 1922). Michael Bliss captured its importance to mankind with the statement “The discovery of insulin at the University of Toronto in 1921-1922 was one of the most dramatic events in the history of the treatment of disease (Bliss, 1996). Eli Lilly and Company introduced the first commercial insulin preparation in 1923, the first rDNA human insulin nearly 70 years later (Chance & Frank, 1993), and the first bioengineered commercial insulin analog in 1996 during the 75th anniversary year for the discovery of insulin (Ross, 1996). Insulin’s rich history is replete with numerous scientific achievements (Steiner, 1977; Brange, 1987; Galloway and Chance, 1994).
Review of Literature

Problems with insulin therapy

The problem of the diabetic, however are from being solved. Insulin-treated diabetics are at risk to develop cardiovascular and kidney problems, and are prone to cataracts and visual impairment, including blindness. Some clinicians feel that these problems occur, not because of insulin therapy itself, but because of the poor blood glucose control obtained by the way in which insulin is administered. They argue that the tissues of an insulin-treated diabetic rarely, if ever, experience physiologic levels of insulin and that a method of delivering insulin physiologically must be developed. Ideally, the insulin should be introduced into the portal circulation to mimic normal pancreatic secretion.

Insulin is a protein and is destroyed by digestion, but clinical trials have suggested that it can be absorbed in the duodenum. Doses 20 times higher than those effective subcutaneously are required. However, efforts are being directed towards the derivation or compartmentalization of insulin in hope of decreasing the dose required. Oral administration of insulin would also eliminate the problem of lipodystrophy that may develop at the injection site. Because of its proteinaceous nature, insulin can cause allergic reactions. Hypersensitivity to insulin is common, but fortunately, most of the dermal reactions are fairly mild and disappear within a week or two after initiation of therapy. Generalized allergic reactions occur much less frequently. Anaphylactic shock has also been reported in conjunction with insulin therapy, but it is extremely rare. It is not always possible to attain good control of blood sugar levels by insulin administrations.

Insulin antibodies are normally found in virtually all patients with in a few weeks of starting injections. If insulin resistance, mediated by elevated levels of antibodies, occurs with conventional therapy, an alternative would be to convert the patient to more purified preparations. Newly diagnosed diabetics treated from the start with
human insulin generally produce fewer antibodies than patients treated with the animal insulin.

*Attempts to reproduce physiological glucose homeostasis*

The Pharmacodynamic effects of insulin are a result of various complex factors, including type of insulin, route of administration, rate of absorption, liver function, glucose concentration, and influence of counter regulatory hormones (Hoffman & Ziv, 1997). Exogenous insulin given to diabetic patients has generally failed to reproduce glucose homeostasis as seen in non-diabetic individuals. This failure occurs because of three principal reasons. The first and perhaps most important reason is that subcutaneous insulin is delivered to the peripheral circulation rather than to the portal circulation (and directly into the liver), which is the physiological route in non-diabetic individuals. Second, absorption from subcutaneous injection sites is slow and variable (± 30-35% dose to dose variability) (Clement, 1999), resulting in the third reason that is the titration of required insulin dosages can be very difficult in the diabetic patient.

As the liver is the primary site of glucose regulation homeostasis, it is generally recognized that insulin delivered into the portal vein is a major determinant of hepatic glucose production. In the non diabetic individual blood glucose concentrations are maintained in a relatively narrow range as the liver takes up glucose in the fed state and releases it into the circulation in needed amounts during fasting (Chipkin et al., 1994). It is important to recognize that hepatic regulation of glucose in the non-diabetic individual is associated with substantially lower insulin concentrations than those required when systemic doses of insulin are given to regulate glucose. In diabetic patients, parenteral insulin treatment frequently produces a peripheral hyperinsulinemia, with insulin eventually reaching the liver at lower concentrations than those associated with direct portal...
delivery. Thus parenteral treatment may deprive the diabetic of the potential for hepatic control of glucose in response to physiological concentrations of insulin delivered via the portal circulation. In addition, peripheral hyperinsulinemia has been linked to hyperinsulinemia and development of atherosclerosis (Chait & Bierman, 1994). Delivery of exogenous insulin by the oral route provides insulin directly to the liver through the portal circulation, more closely approximating what occurs in the non-diabetic individual. Accordingly, treatment with effective oral insulin may provide the benefit of needed hepatic activation while avoiding hyperinsulinemia and its associated long-term complications.

**Association of insulin**

Insulin exhibits a very complex association pattern in the crystalline state as well as in solution. In solution it exists as an equilibrium mixture of monomers, dimers, tetramers, hexamers and possibly some higher associated states dependent on concentration, pH, metal ions and salts.

**Metal ion binding**

Insulin is capable of binding large amounts of divalent metal ions either in the crystalline state or in solution in the slightly acid to slightly alkaline range. At low pH insulin does not bind divalent metal ions like zinc (Chunningham et al., 1955; Schlichtkrull, 1956a). The interaction with zinc requires the formation of hexamers and is strongly pH dependent. The content of bound zinc rises steeply when pH increases from 4.5 to 7.4 (Hallas-Moller et al., 1952). In the pH range 5-7 strong binding of zinc atoms per hexamer of insulin. The involved binding sites have been found to include the alpha amino groups in the insulin molecule (Marcker, 1960; Frank, et al., 1972). However, reported evidence that these sites are most likely the carboxyl groups of the glutamyl residues. The porcine zinc insulin complexes are soluble at neutral reaction when the
zinc/insulin (hexameric) ratio is below 4, but higher concentration of zinc, the solubility decreases (Finger et al., 1967) and at a ratio of 6 \( \text{Zn}^{++}/\text{hexamer} \) complete precipitation of the insulin is observed (Crant et al., 1972).

**Crystals and crystallization**

Porcine and human insulin form geometrically perfect rhombohedral shape but bovine insulin has tendency towards formation of disorted twin crystals often with star like appearance. The crystal structure of human insulin has been found to be nearly isomorphous to that of porcine insulin in the zinc version (Chandhay et al., 1983). The growth of an insulin crystal is anisotropic as insulin is only deposited on three of six crystal faces, the rate being a simple power function of the concentration of insulin in solution.

**Chemical reactions**

The chemical reactivity of the various functional groups in the molecule obviously makes insulin susceptible to transformation. The variety of amino groups of insulin is in a very reactive state at neutral pH values.

**Mechanisms of prolongation**

The basic principle in prolonging the action of insulin is to delay the absorption after subcutaneous injection by altering the solubility of the insulin at physiological pH. Different approaches used were cationic organic compounds added to acid solutions of insulin (The precipitate formed in-vivo are more readily absorbed than preformed complexes), neutral suspensions of insulin combined with basic proteins (E.g. protamine zinc insulin, neutral suspension of insulin complexed with small amounts of zinc ions (E.g lentard type), alteration of the physical state and size of the suspended insulin zinc particles entails variations in the duration of action (Ultra lente
injection), species of insulin of the crystalline zinc insulin suspension (Monotard) and chemical derivation of insulin.

**Absorption**

An acid solution preparation will pass the isoelectric precipitation zone of insulin after injection and high zinc content will impede the redissolution of precipitated insulin and thereby delay the absorption. The high zinc content of acid solution was found to stabilize the insulin (Sahyun et al., 1939). Most likely because of an inactivating influence on contaminating enzymes.

### 3.1.4. Formulations Available

**Protracted Preparations**

Shortly after the introduction of first acid insulin solutions the search for protracted insulin preparations started. The early attempts to prolong the action of insulin included combination with gum arabic (1923), lecithin (1923), oil suspension (1925), other proteins (1925) and cholesterol (1926). However they were unsuccessful due to poor stability of the preparations, pain upon injection or too variable of absorption rate. Subsequently attempts were made to obtain prolonged effect by the combination of insulin with vasoconstrictor hormones, such as adrenaline and vasopressin, but these attempts were also abortive due to great variations in the clinical effect (Dorzbach & Muller, 1971).

The first successful protracted insulin preparation was protamine insulin, introduced in 1936 by Hagedorn and co-workers. The principle was to depress the solubility of insulin at neutral pH using a basic compound. Protamines were among other basic peptides (Histones, globins, etc) found to show the most promising effect. Since the first neutral protamine insulin suspension was not stable it was necessary to dispense this new preparation into two separate vials, one containing a phosphate buffer and the other an acid
solution of protamine and insulin (showing the same timing of action as soluble rapid acting insulins when injected separately). The patient would then prepare sufficient suspension for a few days by injecting buffer into the vial with the acid solution of protamine and insulin.

Later more stable protracted preparations were developed, still including the combination of insulin with foreign proteins. It was not until the introduction of the lente preparations (Hallas-Moller et al., 1951) that protracted insulin preparations without added foreign proteins or synthetic compounds were obtained.

Protamine Insulin

Protamine is the generic name of a group of strongly basic proteins present in the sperm cell nuclei in salt like combination with nucleic acids. Commercially available protamines are made from fish sperm and usually obtained as the sulphate salt. Since protamines emanating from different families, genera and species of fish vary as to peptide composition; it is desirable to specify the family, genus and species of the fish from which the protamine is isolated. Protamines used together with insulin are normally obtained from salmon (salmine) or trout (iridine).

Salmine and iridine are inhomogeneous and have been separated into two and three main fractions, respectively (Ando & Watanebe, 1969). Each of these fractions is probably also heterogeneous as shown for iridine (Ling et al., 1971), but the different peptides having about 30 amino acids residues are very similar in structure. The average molecular weight of protamine has been found to be approximately 4300 for iridine and 4250 for one of the fractions of salmine (Ando & Watanabe, 1969).

Basic residues constitute about two third of all the amino acids residues in protamines resulting in an isoelectric point of above 12. Salmine and iridine belong to the monoprotamines, which contain
arginine as the only basic residue. In addition these protamines contain relatively few other residues predominantly Ser, Pro, Val and Gly.

Being devoid of aromatic amino acid residues, protamines have no UV-absorption in the region 260-360nm, thus any absorption in this range denotes the presence of potential impurities. For example DNA or histones, which contain aromatic residues and are of higher molecular weight.

Protamines were earlier considered non-immunogenic (Kern & Langner, 1939; Jaques, 1949) and the observed immunological reactions to protamine have been attributed to contaminants (Caplan & Berkman, 1976). Allergic reactions due to the protamine content of NPH preparations have, however been reported (Shore et al., 1975; Sanchez et al., 1982) and Samuel concluded that protamines can be immunogenic in man and their use for medical purposes may lead to formation of antibodies (Samuel, 1977; Samuel et al., 1978). Kurtz et al (1983) found evidence that the protamine insulin complex is itself immunogenic, as they showed high prevalence of concomitant circulating antibodies against insulin and protamine in patients treated with protamine containing insulin preparations.

Anaphylactic reactions to protamine sulphate have also been reported (Nordstrom et al., 1978; Moorthy et al., 1980; Knapke et al., 1981; Vontz et al., 1982; Weiler et al., 1985) and the possibility of an allergic reaction to protamine must be considered in patients who are allergic to fish (Knapke et al., 1981). Stewart et al (1984) found a 50 fold increased risk of severe adverse reaction to protamine, if protamine is administered to diabetics receiving protamine containing insulin preparations. The toxicity of protamine has been reviewed by Harrow (1985).
Protamine Zinc Insulin (PZI)

The first stable neutral suspension was developed by Scott and Fisher (1936), who discovered that a surplus of protamine and zinc salt in small quantities (2μg zinc/IU) could stabilize the neutral protamine insulin. This protamine zinc insulin (PZI) has a much more prolonged effect, which may last for up to 72 hours (Colwell, 1947). The dissolution of insulin before absorption is presumably due to degradation of the protamine by the fibrinolytic tissue enzymes (Hagdorn et al., 1936; Hagedorn, 1946; Bang, 1946; Brunfeldt & Poulsen, 1953). The protaminase activity of serum is inhibited by zinc (Brunfeldt & Poulsen, 1953), which may explain different timing of PZI and NPH.

PZI made according to United States or European Pharmacopoeias contains amorphous as well as crystalline protamine zinc insulin. Freshly prepared PZI contains mainly amorphous precipitate, which will gradually be transformed to crystalline particle upon storage, leading to a more protracted effect. Physically stable PZI preparations can be made either by avoiding the zinc binding phosphate buffer (resulting in intermediate acting amorphous PZI) (Schlichtkrull, 1958) or by crystallizing the protamine insulin before the surplus of protamine and zinc are added (resulting in long acting crystalline PZI).

NPH (Neutral Protamine Hagedorn)

A stable PZI modification, NPH (Netural Protamine Hagedorn) also called as “Isophane Insulin” was developed by Krayenbuhl & Rosenberg (1946) at Nordisk insulin laboratorium. They found that insulin and protamine brought together in isophane proportions (the condition in which neither insulin nor protamine is found in excess) at neutral pH, in the presence of a small amount of zinc and phenol and/or phenol derivative (cresols) will form amorphous precipitate, which is gradually transformed into tetragonal oblong crystals.
limited at the ends by pyramidal faces. At pH 7.3 insulin and salamine co-precipitate in a 5:1 molar ratio corresponding to about 0.13 mg protamine per mg insulin (Simkin et al., 1970).

The following conditions are necessary for rapid and complete crystallization of protamine insulin: the protamine, insulin and auxiliary substances must be reasonably pure and the proportion between protamine and insulin nearly isophane. The isophane ratio varies with different protamine and insulin qualities and species as well as with pH, temperature, content of zinc and auxiliary substances. Zinc in a concentration of approx, 0.2µg/IU is necessary for the preparation of the tetragonal crystals as is the presence of phenol or phenol derivatives, m-cresol is more suitable for the crystallization than the corresponding o-or p-derivatives (Krayenbuhl & Rosenberg, 1946) or phenol (Fullerton & Low, 1970).

Insulin-salmine crystals contain insulin and protamine combined in a complex in a molar ratio of 8.5 to 1 (Fullerton & Low, 1970). It is assumed that protamine is to be found in the interstices between hexamers but not as an ordered component of the crystal lattice (Simkin et al., 1970; Hodgkin 1974).

Zinc is present in the same amount as in rhombohedral crystals (2 atoms of zinc per hexamer) together with about 0.5 x 10⁻³ mol phenol (or phenol derivative) per gram of protamine insulin corresponding to 22 moles of phenol per hexamer (Krayenbuhl and Rosenberg, 1946). Suspensions of such crystals are stable in the absence of protamine-degrading proteolytic enzymes, such as pancreatic enzymes, which may be present in impure insulin.

Graham & Pomeroy (1984) found marked differences in duration of action between different brands of NPH largely due to variations in crystal size and shape rather than to differences in insulin species.
**Insulin Zinc Suspensions (Lente Insulins)**

The Lente insulins were developed in the NOVO laboratories by Hallas-Moller & co-workers (1952), Hallas-Moller (1956) elucidated the influence of zinc ions on the timing of insulin preparations. They showed that insulin preparations with protracted effect could be obtained by addition of small amounts of zinc ions provided that the preparations had a neutral pH and that no interfering (zinc binding) ions, like phosphate or citrate were present.

Furthermore they showed that the degree of protraction at the same zinc concentration depended on the physical state of the suspended insulin particles, amorphous insulin particles having a shorter time of action than crystalline insulin particles.

This led to the introduction of three new protracted insulin preparations all containing approximately 80 μg zinc/ml (40 IU/ml): semilente containing only amorphous insulin particles, ultralente containing only crystalline insulin particles and lente containing a 3:7 mixture of amorphous and crystalline insulin particles. Bovine insulin was originally chosen for the crystalline part and porcine insulin for the amorphous part. Later monotard, having a composition like lente with the exception that monotard contains porcine or human insulin only, and lente preparations, containing only insulin of bovine origin, have been introduced. Preparations containing insulin derivatives in the amorphous part have been investigated clinically (Bottermann et al., 1980).

The lente insulins were originally developed in the search for preparations able to cover diabetic's insulin requirement with one daily injection. Mixing the three types of lente preparations in different ratios may make adjustments. In modern clinical treatment individual mixtures of intermediate acting and rapid acting preparations are often given more than twice daily. A regime where the basal insulin requirement is covered by ultratard, while actrapid
is used to cover meals have been described by Phillips et al (1979) and Ward et al (1981).

In the lente preparations a certain proportion of the total amount of zinc is present as zinc ions bound within the suspended insulin particles, whereas the rest is present as free zinc ions in solution. Schlichtkrull (1958) established an empirical relationship between the amount of bound zinc ions (in number of zinc atoms per insulin hexamer), the concentration of free zinc ions and the concentration of hydrogen ions (constant temperature, pH around 7.3). At the constant pH value of the preparations this means that at constant free zinc concentration, the amount of bound zinc in mg/100 IU insulin (Proportional to the number of zinc atoms per insulin hexamer) will be constant independent of total insulin concentration.

A concentration of free zinc of approximately 0.05mg/ml at pH 7.4 was chosen for the original lente series. This lead to an amount of bound zinc of approximately 0.09 mg/ 100 IU of insulin.

Calculations of bound and total zinc based on a concentration of free zinc of 0.05 mg/ml are reported for three different insulin strengths. The total zinc content relative to insulin (in mg/100 IU) is adapted to the various strengths in order to maintain constancy of the chemical composition of the solid phase and of the suspension medium, aiming at an unchanged protracted effect.

The composition of lente preparations with respect to other auxiliary substances are described at length by various reports, one should be emphasized that, for example, phenol is unacceptable as preservative, since its presence leads to a change of the physical state of the insulin particles and thus probably a change of the dissolution and timing properties.

In the manufacture of ultralente or the crystalline part of lente and monotard, the insulin is dissolved in acid, sterilized by filtration and thereafter crystallized. After the crystallization, pH is adjusted to 7.4
and the correct concentrations of insulin, zinc and other auxiliary substances are established. The crystals formed during the crystallization are of the 4Zn-insulin structures, but X-ray studies have shown that complete conversion into the 2Zn-insulin structures takes place during dilution and pH adjustment of the crystal suspension.

In order to obtain a constant and narrow size distribution of the insulin crystals a special seeding technique has been used as described by Schlichtkrull (1957b). This is advantageous because the timing of the preparations is to some degree dependent on the size of the crystals. In the manufacture of semilente or the amorphous part of the lente preparations, the insulin is dissolved at acid pH and after sterile filtration the correct pH and zinc concentration are established. This leads to a precipitation of insulin as amorphous particles.

The size of the individual amorphous particles is about 1 µm. Microscopically, the amorphous particles are seen to form loose aggregates. This flocculation is important for the physical stability of the preparations. Formation of lumps or flakes due to inappropriate storage has been shown to decrease with increased flocculation. Similar results have been reported by Haines & Martin (1961) for other suspensions.

The original lente preparations were prepared using zinc chloride as the source of zinc ions but, as the amount of zinc ions present are the important factor, other zinc salts (e.g. acetate) are also reported to be useful. A detailed survey of the physical chemistry of the lente insulins reference is made reported by Schlichtkrull et al (1975).

Biphasic Preparations

When using intermediate-acting insulin preparations the initial insulin effect is often too slight. A stronger initial effect can be obtained by mixing rapid or short acting preparations with
intermediate-acting preparations. The need for a stronger initial effect led to the search for stable mixtures of rapid acting (dissolved) and intermediate to long acting insulin resulting in the development of Rapitard (Biphasic Insulin Injection) (Schlichtkrull, 1959; Schlichtkrull et al., 1965).

Rapitard contains 75% crystalline bovine insulin suspended in a solution of 25% of mainly porcine insulin. The rationale for this preparation is the utilization of the difference in solubility between porcine and bovine insulin at specific values of pH and zinc concentration. Due to the low solubility of the bovine insulin, only a small amount of the dissolved insulin is bovine.

The insulin crystals are prepared in the same way as the ultralente and the crystalline part of the lente preparation. Crystallization in the presence of 7% sodium chloride leads to crystals containing 4 zinc atoms per insulin hexamer. After crystallization the preparation is diluted at constant pH (5.5) to the final concentration of insulin and auxiliary substances (i.e. 0.7% w/v of sodium chloride). This leads to a crystal transformation from crystals containing 4 zinc atoms per insulin hexamer to crystals containing 2 zinc atoms per hexamer. The crystal suspension is later adjusted to pH 7 and mixed in the correct proportion with the separately prepared neutral solution of insulin. Rapitard is physically stable, the proportion of insulin in solution remaining constant during shelf life.

Two biphasic preparations have been introduced based on isophane insulin like initard and mixtard. Initard is a 1:1 combination of regular and NPH insulin, and mixtard prepared by adding 3 parts of regular insulin to 7 parts of NPH insulin. These mixtures are not physically stable, as some of the dissolved insulin is transferred to the solid phase. In mixtard, only about half the amount of the added regular insulin is recovered in solution (Galloway et al., 1982), whereas in initard two thirds of the added regular insulin can be detected in the supernatant. A biphasic insulin preparation utilizing
the higher solubility of the insulin derivative, des-Phe^{B1}-insulin, as compared to that of the parental insulin has been introduced as optisulin depot during 1980s (Zoltobrocki et al., 1980).

Other Types of Protracted Preparations

Surfen insulin preparations of intermediate and long duration of action have been widely used in Germany. The prolonged action of these preparations is based on formation of slightly soluble complex between insulin and the synthetically produced substance, 1, 3-bis (4-amino-2-methyl-6-quinolyl) urea (surfen) at neutral reaction (Lautenschlager et al., 1937; Umber et al., 1938). The intermediate acting depot-insulin with 4.2 µg surfen / IU is an acid solution from which the surfen insulin complex precipitates as amorphous particles after injection. Komb-Insulin is a mixture of one part acid regular insulin and two parts depot-insulin, which is a neutral suspension of crystalline and amorphous surfen insulin in the ratio 3:1. Several cases of allergic reactions to surfen have been reported (Kulpe, 1958; Forck et al., 1975; Goerz et al., 1981) and the incidences of these reactions are reported to be increased (Goerz et al., 1981).
3.1.5. Novel Alternative Methods of Insulin Replacement Therapy

In an attempt to overcome the inconvenience and discomfort of parenteral treatment, various non-invasive alternatives to injectable insulin have recently been evaluated which involve oral (enteric) delivery of native insulin, oral (enteric delivery of modified (conjugated) insulin, oral (buccal) spray, nasal spray, inhaled (lung alveoli) etc.

Oral Insulin Development

Past history indicates that development of effective oral insulin is a challenge of significant proportions. The path of drug development is littered with failed attempts to create a viable oral insulin formulation. Previous attempts have consisted primarily of formulation engineering that resulted in a relatively poor absorption from the gastrointestinal tract and substantial variability in the amounts absorbed both within and among subjects. These barriers are essential to overcome for any oral insulin product to be successful. Bioavailability must be within the range of economic viability, that is low bioavailability may be over come to a certain extent by administering very large amounts of drug, however there are limits beyond which the cost of the drug may be prohibitive to practical use. Concomitant with low bioavailability is dose-to-dose variability in absorption, resulting in serious difficulty in accurate titration of drug effect. The ability to titrate insulin doses in a reasonably accurate and reproducible manner is essential to successful management of diabetes. A company named NOBEX has directed development of conjugated oral insulin towards overcoming the challenges of bioavailability and titratability.

Many formulation approaches utilizing features such as penetration enhancers, enzyme inhibitors, and various forms of enteric coatings have been employed in order to improve the oral bioavailability of native insulin. While some formulations have resulted in modest
improvements in absorption, bioavailability has not yet been considered adequate to make this approach viable.

Administration of insulin to the buccal mucosa appears to be promising as an alternative to injectable treatment and is currently in phase-II testing (Gordan, 2002). In addition to a major disadvantage of buccal delivery is that insulin administered by this route enters directly into the peripheral circulation, resulting in peripheral hyperinsulinemia similar to that seen with injectable insulin.

While delivery of insulin through the nasal mucosa by a spray device appeared initially to hold promise, the formulations utilized resulted in unacceptable levels of intra patient, dose to dose variability of insulin absorption and nasal mucosal irritation. Similar to insulin delivered through the buccal mucosa, nasal administration also routes the insulin directly into the peripheral circulation.

Delivery of insulin to the alveoli of the lungs has been under development by several sponsors and appears promising. The usefulness of this product may be limited by the requirement to master the operation of the delivery device, the potential for lung irritation with long-term use, and with impaired delivery in patients with lung disorders. As with buccal or nasal mucosal delivery, insulin absorbed through lung alveoli enters directly into the peripheral circulation, therefore providing no advantage over injectable insulin other than elimination of the discomfort of needle puncture.

Oral enteric delivery of insulin conjugated with amphiphilic oligomers holds substantial promise towards achieving the goal of non-invasive delivery of insulin in a manner that closely duplicates the physiological secretion of insulin in the non-diabetic individual. The chemistry and pharmacology of this product is described below.
Conjugated Insulin: Chemistry and Pharmacology

The chemical structure of NOBEX conjugated insulin is a single oligomer (PEG\textsubscript{7-9} + hexyl) is covalently attached at the lysine 29 site on the B-chain of recombinant human insulin. This conjugated insulin is approximately two to eight times (pH-dependent) as soluble in water as the unconjugated molecule, this allows the conjugate to be formulated in a variety of excipient combinations that enhance absorption across gastrointestinal mucosa. In receptor binding and cell based receptor activation assays, the conjugated insulin has been found to be approximately 70% as active as native insulin. In addition, the oligomer imparts significant stability against gastrointestinal enzymes, with degradation delayed between two to ten times that of native insulin, depending on the enzyme studies. Because, of the substantial hepatic uptake of portal insulin and the resulting hepatic influence on glucose homeostasis, traditional pharmacokinetic analyses are of limited relevance for evaluating absorption of this conjugate. However, studies in normal, fasted dogs suggest that bioavailability relative to direct portal infusion of insulin is approximately 8%. No bioavailability studies involving humans are available till date for such insulins, but data from phase-I and II clinical trials suggest that the apparent bioavailability of the current formulation is approximately 5%. It is reported that accurate assessments of the amounts of conjugated insulin required to manage hyperglycemia to be obtained from repeat dose titration studies in diabetic patients (Gordon, 2002).

3.1.6. Standardization and Chemical Methods

Immunogenicity of insulin

Awareness of Immunological side effects of insulin therapy became apparent during the later forties, and it was later established that virtually all insulin treated diabetics had insulin antibodies (Berson et al., 1956; Berson & Yallow, 1959; Berson & Yallow, 1964). In some
patients the presence of high levels of antibodies caused severe insulin resistance due to the elimination of insulin as antigen-antibody complexes (Berson & Yalow, 1960). In other patients the antibodies were found to change the control of glycemia by firstly capturing the injected insulin, thereby reducing its biological effect, and later releasing it from the complexes, which could lead to hypoglycemia (Berson & Yalow, 1960). These side effects were thought to be inevitable until the introduction of the highly purified insulins in the early seventies.

Besides inducing insulin antibodies, insulin preparations containing detectable amounts of contaminants such as PP, VIP, glucagons and a component, can induce antibodies against all these substances in insulin treated diabetics (Bloom et al., 1979), Villalpando & Drash, 1979; Heding et al., 1980) The biological significance of these antibodies is unknown. Radical elimination of the contaminants from the insulin has completely abolished the induction of such antibodies. When patients are transferred from conventional crystallized insulin to MC insulin a rapid decrease in the level of antibodies against the contaminants is observed (Heding et al., 1980). The mentioned antibodies all belong to the IgG class of immunoglobulin and bind and neutralize their antigens.

Antibodies of the IgE class specific for PP, a component and insulin may also be induced by insulin preparations (Falhot et al., 1983). The IgE1 against insulin causes allergic manifestations. Falholt (1982) has reported insulin purified to MC specifications do not induce IgE1, irrespective of the insulin species.

During the development of MC-insulin aiming at reducing the formation of insulin antibodies (Schlichtkrull et al., 1970), an animal model was developed to control the various batches of insulin. The rabbit was found to be the most suitable species as it develops insulin antibodies with similar capacities and affinity binding constants as do diabetic patients (Schlichtkrull et al., 1972).
a considerable variation between the responses in rabbits to the same immunological stimulus but, irrespective of its shortcomings, the method is useful as a test for immunogenecity whenever new preparations or new technology for the production of insulin is introduced. In this rabbit model monocomponent porcine as well as human insulin exhibit none or very low antibody formation and the two insulin species cannot be distinguished from each other. In clinical trials, however the immunogenecity of human insulin has, in several studies, been found to be lower than that of porcine insulin. After 6 months treatment of 102 newly diagnosed diabetics with either porcine or human monocomponent insulin Schernthaner et al (1983) found IgG insulin antibodies in only 14% of the patients treated with human insulin, but in 29% of those treated with porcine insulin. The antibody titers were also significantly lower in the patients treated with human compared to the patients who received porcine insulin. In a multicentre trial Heding et al (1984) compared the two insulin species in 135 newly diagnosed diabetic children and concluded that human monocomponent insulin has a lower immunogenecity than porcine insulin of the same purity during the first year of insulin treatment. In a long term study Luyck et al (1986) found that the percentage of patients who remained antibody free after 12-21 months of treatment was 67-75% in the group treated with monocomponent human insulin and only 25-43% in the one receiving monocomponent porcine insulin and the insulin antibody titers, when present, were lower in subjects treated with human insulin.

Velcovsky & Federlin (1984) also observed lower antibody concentrations in patients treated solely with human insulin compared to a group treated with porcine insulin and it was found that four diabetic patients with delayed-type allergy to animal insulin could be treated with human insulin without any problems. They
concluded that the introduction of human insulin represents an important advance from the immunological point of view.

**Mechanism of Prolongation**

The basic principle in prolonging the action of insulin is to delay the absorption after subcutaneous injection by altering the solubility of the insulin at physiological pH. The different approaches relate to the following are described below:

a) Cationic organic compounds, added to acid solutions of insulin. These will bind the insulin at the neutral reaction of tissue fluids making heavily soluble complexes. Examples of complexing agents are surfen and globin. The precipitates formed in vivo are more readily observed than preformed complexes.

b) Neutral suspensions of insulin combined with basic proteins. Examples are protamine zinc insulin (amorphous or crystalline variety) prepared with surplus protamine and isophane (NPH) having a stoichiometric ratio between insulin and protamine.

c) Neutral suspensions of insulin complexed with small amounts of zinc ions. Examples of which are, the lente type preparations with 2μg zinc per IU in U-40 preparations and crystals made of bovine insulin containing as little as 0.3μg zinc per IU (Rapitard).

d) Alteration of the physical state and size of the suspended insulin-zinc particles entitles variations in the duration of action. Small amorphous particles (Semi-lente) have a slightly slower effect than regular insulin whereas crystalline particles (Ultralente) have a substantially more retarded effect, which again will vary with the size of the crystals, which emphasizes the importance of uniform size distribution from batch to batch.

e) Species of insulin of the crystalline zinc insulin suspensions. The duration of action of the porcine crystals is somewhat shorter than
that of the bovine variety. An example is lente (bovine crystal phase) and monotard (Porcine crystal phase).

f) Chemical derivation of insulin is the other method of prolonging insulin action. Hallas-Moller (1945) coupled the amino groups of insulin with phenylisocyanate (Iso-insulin). Schilichtkrull (1958) observed that special heat treatment of zinc insulin crystal at pH 5.5 (but not at pH 7) produced a substantial enhancement of the retardation. No explanation was found at that time, but it has since become apparent that a chemical reaction between hexamers in the crystals takes place under those specific conditions resulting in the formation of covalent dimers of insulin. The covalent link between monomers located in different hexamers will cross-link the hexamers within the crystals and thereby decrease the solubility of the crystals. Formation of only a few percent of dimer will cause the substantial change in the solubility and consequently timing. A preparation of this type has been successfully used in the treatment of streptozocin induced diabetic rats (Rasch, 1979).

3.1.7. Analysis and Standardization of Insulin

Assessing the potency of insulins by a bioassay implies a certain risk of variation in the strength of the final insulin preparations. The use of bioassay was mandatory in the years following the discovery of insulin, since the composition and purity of insulins produced could vary considerably from batch to batch. Today when insulins can be produced with a uniform and very high purity, it has become possible to reduce the potency variation between batches of pharmaceutical insulin preparations by calculating the potency of insulin by accurate chemical methods of analysis such as Kjeldahl nitrogen determination (Pingel et al., 1982) or HPLC (Kroeff and Chance, 1982). Quantitation by HPLC of insulin and related substances in bulk insulin and in insulin preparations has been found to give an estimate of the potency that is in good agreement.
with that obtained by the rabbit blood sugar assay (Smith et al., 1982) and the mouse blood sugar assay (Fisher & Smith, 1986). The HPLC method was found to reduce analysis time and to give more reproducible results. A bioassay may be used for control and the potency estimate should be consistent with the estimate obtained by the chemical methods. The biological potency of monocrytalline insulin whether porcine, bovine or human has been found to be the same on molar basis. 168.10^6 IU/mol corresponding to 184 IU/mg (Pingel et al., 1982) determined by the mouse convulsion assay.

Purity

Proper characterization of the purity of insulin requires a combination of analytical methods based on various principles (Jorgensen et al., 1982). The four most important analytical principles for the characterization of insulin with respect to purity are the classical biochemical methods of gel filtration (fractionation by molecular size) and disc electrophoresis (fractionation mainly by charge) to which have been added the highly sensitive methods of radioimmunoassay (RIA) and, recently, the high performance liquid chromatography (HPLC) methods. Improved, modifications of HPLC techniques are expected to replace gel filtration and disc electrophoresis in the future.

Gel Filtration

In 1967 it was shown by means of gel filtration that commercial insulin, purified solely by crystallization, contained impurities with a higher molecular weight than that of insulin (Steiner, 1967). These impurities were later identified to be mainly proinsulin, proinsulin intermediates and covalent insulin dimer (Steiner et al., 1968). Since, then several gel filtration systems for the analysis of insulin have been described (Rolando and Torroba, 1972, Schlichtkrull et al., 1974, Fisher & Porter, 1981). Common to nearly all methods is the use of Bio-Gel P-30 (Bio-Rad Laboratcrries) or Sephadex G-50
(Pharmacia Fine chemicals) and 1-3 M acetic acid as eluent. In this medium insulin is fully disassociated. Gel filtration chromatograms of porcine insulin. That is crystallized insulin show three distinct peaks a, b and c. The a-component comprises high molecular weight substances (MW more than 25,000) derived from pancreatic tissue proteins. Its concentration is reduced by crystallizations, but small amounts are still present in 5 times crystallized insulin, since antibodies against a component are detectable in nearly all patients treated with insulin of this purity (Heding et al., 1980). The b-component contains proinsulin and related substances, which are removed to only a slight extent by crystallizations. Finally, the c-component comprises insulin and derivatives of insulin with practically, the same molecular size (insulin ethyl ester, arginine insulin, deamidated insulin, etc) (Schlichtkrull et al., 1974).

Adequate care is essential should be taken when analyzing insulin preparations by gel filtration to avoid possible misinterpretations due to the preservatives of the preparations (phenol, m-cresol or methyl paraben). These substances absorb UV light strongly at the wavelength used for detection of insulin (275-280 nm) resulting in the largest peak of the chromatogram. An example of such a misinterpretation has been described by Schlichtkrull (1977a). Insulin isolated from preparations still contains traces of preservatives, which will lead to peaks in the chromatograms. As the preservatives are of low molecular weight and tend to adsorb to the gel, they are eluted after the insulin peaks. Gel filtration of insulin using HPLC technique has been described by Welinder (1980).

Disc Electrophoresis

Since the introduction of disc electrophoresis in polyacrylamide gels by Ornstein (1964) and Davis (1964) and Mirsky and Kawamura's (1966) subsequent application of this method for the analysis of insulin, it has been widely used for the characterization of insulin.
purity (Tjoe and Wacker, 1972, Schlichtkrull et al., 1974, Krause and Beyer, 1975, Kasama et al., 1980, Fisher & Porter, 1981). Although various electrophoretic systems are used by the different authors, they are all modifications of the original system by Davis and Ornstein, operating at a slightly alkaline pH (8-9). Variations in acrylamide concentration, load per tube, content of dissociating agent (urea), and dye used for staining have been described. One of the most reproducible methods allows application of 100-200 μg of insulin per 5 x 50 mm tube containing 1 ml of gel (Schlichtkrull et al., 1974). At higher loads the insulin and monodesamido insulin are no longer distinguishable as two separate bands.

Staining with Amidoschwarz in combination with diffusional destaining in 3% acetic acid is among the most reproducible methods for visualizing protein bands in polyacrylamide gels. Destaining by electrophoresis cannot be recommended, as weak bands may be removed by this method. A slightly more sensitive stain is coomassie Brilliant Blue G250, as used by Diezel et al (1972), but meticulously standardized conditions during the staining procedure are required to obtain reproducible results. Therefore, staining with Amidoschwarz is more convenient for routine purposes.

A rough quantification of the bands can be made by comparing with gels containing known amounts of the impurities added to monocomponent insulin. Another method is to compare with a series of gels to which varying amounts of insulin have been applied as a protein standard, as prescribed in the British Pharmacopoeia (1980). Because of variation in ability to bind the dye and variation in band sharpness between the different impurities the latter method is less accurate than the former.

*Radio-Immuno Assay (RIA)*

Radio-immuno chemical methods are by far the most sensitive used in the evaluation of insulin purity with a detection limit in the range
of 1-500 ppb by the weight (1 ppb = 1 part impurity in $10^9$ part of insulin). RIAs are two thousand to one million times more sensitive than the other methods described in this chapter (Detection limits about 0.1%). RIAs have proved their great potential in detecting trace amounts in insulin of other polypeptide hormones originating from the extraction of the pancreatic glands.

Shortly after the discovery of proinsulin, the precursor of insulin, in 1968, it was found that commercial insulin preparations contained about 2% of proinsulin, and during the following years RIAs were developed for bovine and porcine proinsulin and C-peptide (Yip and Logothetopoulos, 1969, Heding et al., 1974). The proinsulin RIA was the first of its kind used to evaluate insulin purity.

Proinsulin is composed of the insulin moiety and the connecting people. The sequence of the latter is highly variable between species. This allows the establishment of species specific proinsulin RIAs. Two methods have been used to eliminate interference from insulin. The anti-proinsulin serum is passed through a column of immobilized insulin which removes insulin antibodies. The possibility of insulin interference is thus eliminated, and labeled proinsulin can be used as tracer (Yip and Logothetopoulos, 1969; Aaby 1979)

The anti-proinsulin serum is used without pretreatment and insulin interference overcome by using a tracer that cannot be displaced by insulin, such as 125 I-Tyr-C-peptide (Heding et al., 1974). C-peptide contains no site for labeling with 125 I. Tyrosine is therefore coupled to the N-terminal amino acid the C-peptide to yield Tyr-C-peptide, which is then labeled with 125I (Markussen et al., 1970).

In addition to molecules containing the intact C-peptide moiety, the proinsulin RIAs may detect molecules containing fragments of the C-peptide. Thus, a better description of the material measured is given by the designation PLI (Proinsulin-Like Immunoreactivity), indicating
that the trace contaminant in insulin may not only be proinsulin, but also other compounds with sequences partially identical to that of proinsulin.

The PLI in insulin is attributable to several impurities, consequently, standardization of reagents and methodology is necessary in order to obtain reproducible estimate of the PLI content in insulin (Damgard and Kruse, 1982; Kruse et al., 1984). Shortly after a RIA for Glucagon-Like Immunoreactivity (GLI) became available analysis for GLI in insulin samples was introduced (Unger et al., 1970; Heding, 1971). One of the major problems of this RIA is caused by the sensitivity of glucagons to proteolytic enzymes. Great care must be taken to avoid false estimates stemming from enzyme contamination leading to degradation of standard, tracer or the sample GLI. To minimize the problem a proteinase inhibitor, such as aprotinin, is added to the buffer to inhibit proteolytic degradation. During the last decade new polypeptide hormones have been discovered to be present in the pancreas. This led to the application of additional RIAs for the characterization of insulin purity, namely for Pancreatic Polypeptide (PP) (Chance et al., 1979), somatostatin (Parel & Reichlin, 1979; Tronier and Larsen, 1982) and Vasoactive Intestinal Peptide (VIP) (Fahrenkrug & Schaffalizky, 1977; Bloom, 1979). Thus at least five different RIAs have been used for the characterization of insulin.

The level of some of the hormonal contaminants in insulin has been measured and found to vary greatly from brand to brand (Bloom et al., 1978; Fitz-Patrick & Patel, 1981; Mizuno et al., 1980). Sutcliffe and Bristow (1984) reported that the PLI content in commercial bovine insulin formulations ranged from 0.23 to 4.8% for conventionally purified insulins, whereas the content in highly (chromatographically) purified bovine insulin varied between less than 1 ppm and 1160 ppm (0.1%). The contents of different hormone contaminants need not be correlated, as different purification
methods may yield completely different compositions of the hormonal contamination (Jorgensen et al., 1982). Therefore it is unjustified to use a single contaminant as a common denominator of purity. A more comprehensive radio immunochemical characterization should be used to evaluate purity.

Typical examples of the contaminant content of twice crystallized and monocomponent (MC) insulin. It shows that careful application of modern purification technology can reduce the content of contaminating polypeptides in insulin to a level at or below the detection limit of the sensitive RIAs.

*High Performance Liquid Chromatography (HPLC)*

The most recent method for the analysis of insulin is HPLC. In particular the reverse phase mode of HPLC, with a non polar matrix as bed and a buffered mixture of water and organic solvent as eluent, has revolutionized polypeptide analysis by combining high selectivity and short time of analysis. Separation of bovine and porcine insulin was the first important application of HPLC in insulin chemistry (Biemond et al., 1979; Damgaard & Markussen, 1979; Dinner & Lorenz, 1979), followed by the separation of human insulin from the two other insulin species (Terabe et al., 1979). HPLC methods for separating several species including chicken, rabbit, sheep and horse have since been published (Ohta et al., 1983; Rivier & McClintock, 1983). The introduction of HPLC means that high sensitivity species analysis of insulin preparations can now be performed in less than one hour. The determination of monodesamido insulin formed by acid hydrolysis during extraction of insulin has traditionally been performed in a semi quantitative manner by disc gel electrophoresis. A number of HPLC methods for the quantitation of monodesamido insulin have been published, and in most cases the species composition and monodesamido content can be determined in the same analysis (Szepesi & Gazdag, 1981; Lloyd, 1982). Lloyd &
Corran (1982) have described an HPLC method capable of separating bovine, porcine and human insulin and their respective monodesamido derivatives in the same analysis. In contrast to the number of methods described for species analysis and determination of monodesamido insulin, few publications have described the use of HPLC for quantitation of impurities and insulin derivatives in insulin preparations. HPLC profiles of porcine insulin (Jorgensen et al., 1982; Welinder, 1984), recombinant as well as semi synthetic human insulin (Chance et al., 1981a; Markussen et al., 1982) have appeared.

HPLC of conventional crystallized insulin has shown that insulin derivatives with virtually the same molecular weight as insulin, e.g. mono arginyl-insulin, monodesamido insulin and ethyl ester, can be determined under isocratic conditions i.e. using a constant eluent composition throughout the elution. High molecular weight compounds, such as proinsulin, proinsulin intermediates and covalent insulin dimers, need gradient elution with increasing elution strength in order to be analyzed with reasonable sensitivity in samples of insulin (Jorgensen et al., 1982). Welinder et al (1986) have reviewed the literature concerning reversed-phase HPLC of insulin and investigated the influence of column geometry and eluent composition on resolution and recovery, considering the increasing use of HPLC in insulin analysis. It is without doubt that HPLC analysis to remain as one of the fundamental methods for species analysis and characterization of insulin purity in the future.

Bioassay Methods

Several in vivo and in vitro bioassay methods for insulin have been developed. Only the in vivo bioassays based on the hypoglycemic effect of the insulin after injection into an experimental animal will be discussed below, since only these are recognized by health authorities for control of the biological potency of insulin for
therapeutic use. The methods differ primarily in the species of animal employed, in the experimental design, and the procedure for handling the data.

During 1922-1926 bioassay methods for insulin were developed, based on the measurement of the hypoglycemic response in rabbits and the convulsive response in mice, in order to assess the potency of the hormone relative to an international standard. The classical bioassay methods, which contributed to the establishment of biometry, are still the basis for the pharmacopoeial methods for determination of insulin potency.

The rabbit blood sugar assay using a crossover design was first suggested by Marks (1925). Since then there have been many publications recommending minor modifications to the original rabbit assay (Smith, 1969). Simultaneously with the development of the rabbit blood sugar assay, work was undertaken to utilize the insulin induced convulsions in mice first observed by Frase (1923). Hemmingsen & Krogh (1926) and Trevan & Bock (1926) found that the percentage of mice responding increased with increasing dosage, and this led to the establishment of the theoretical basis for quantal response bioassays. Many studies have been carried out since the precision and reproducibility of the mouse convulsion assay (Smith, 1969; Stewart 1974). Later, Eneroth & Aahlund (1968, 1970a, 1970b) have developed a mouse blood glucose twin cross-over assay.

Several papers have been published showing that the rabbit blood sugar assay, the mouse convulsion assay and the mouse blood glucose assay give essentially similar bioassay results (Miles et al., 1952; Bangham & Mussett, 1959; Ashford et al., 1969; Bangham et al., 1978). However, an analysis of more than 500 rabbit blood sugar assays by new multivariate statistical methods showed that the potencies based on blood sugar responses $\frac{1}{2}$, 1 and 2 $\frac{1}{2}$ hours
after the injection of pure porcine, bovine and human insulins relative to the present mixed species standard showed significant variation depending on the blood sampling times (Volund et al., 1982; Pingel et al., 1985). Porcine and human insulin potencies decreased by 12% and 18% respectively, from the half hour to the 2 ½ hour response. Whereas bovine insulin potency increased by 9%. Since the standard is approximately an even mixture of porcine and bovine insulin these results could be due to porcine and human insulin having a quicker onset and shorter duration of hypoglycemic effect than bovine insulin. This was confirmed in the assays of porcine relative to bovine insulin and by direct comparison of mean blood sugar curves. It was concluded that the rabbit blood sugar assay is invalid when the test and standard insulin have a different species composition. Hence, pure species insulin standards are needed for this assay.

In the mouse convulsion assay no difference in the effect of porcine and bovine insulin has been demonstrated (Pingel et al., 1982). Thus this bioassay system can be used whether the test and standard preparation are of the same or of different insulin species.

3.2. Role of trace elements in Diabetes Mellitus

3.2.1. Chromium

Chromium [as Cr (III)] is an essential trace mineral found in foods and supplements in the oxidized form, Cr (III), the most stable form of chromium (Curran, 1954). Chromium is present in small amounts in many foods commonly consumed in the U.S. (Anderson et al., 1992) and is considered one of the least toxic nutrients (Xi et al., 2001). A significant portion of the chromium present in foods is believed to originate from the soil and from external sources during growing, processing, preparation, fortification and handling (Anderson et al., 1992). Currently the Institute of Medicine recommends that adult men aged 14 to 50 years consume 35 µg of
chromium daily and adult women aged 14 to 50 years consume 25 mcg of chromium daily. Those recommendations are reduced by 5 µg daily for individuals over the age of 50 years.

Anderson and Kozlovsky (1985) estimated that the 7-day average chromium intake by 10 adult males was 33 ± 3 mcg (range 22 to 48 µg per day), and the estimated intake by 22 adult females was 25 ± 1 µg (range 13 to 36 mcg per day). Those estimates correspond with approximately 14 µg and 16 µg of chromium per 1000 kcal of dietary energy intake for males and females, respectively. In another study of the chromium content of self-selected diets, in which the chromium contents of foods was directly measured, the average chromium concentration of diets selected by 8 adult males was 18.6 µg per 1000 kcals, and the average chromium concentration in diets selected by 11 adult females was 12.5 mcg per 1000 kcals (Anderson et al., 1992). The self-selected daily chromium intake was determined to be 38.8 ± 6.5 µg per day for the males and 23.1 ± 2.9 µg per day for the females (Anderson et al., 1993). When corrected for self-selected energy intake, self-selected daily chromium intake was determined to be 54.1 ± 7.2 µg per day by males and 28.7 ± 3.1 µg per day for females (Anderson et al., 1993).

Recently, Juturu et al (2003) reported estimates of the intake of chromium based on food servings consumed by free-living male and female adults and children. The consumption of chromium from food sources is shown to be less than 20 µg per day (Juturu and Komorowski, 2003). Previously Kumpulainen et al (1979) have reported dietary consumption to be lower in the range of 25 to 224 µg.

Absorption and Bioavailability of Chromium

Trivalent chromium (Cr (III)) is present in many foods and is ingested as a normal part of the daily diet. However, only about 0.4 to 2.0% of dietary trivalent chromium is absorbed by laboratory animals and
humans, predominantly in the small intestine (Gammelgaard et al., 1999) whether from food (Anderson and Kozlovsky, 1985; Anderson et al., 1983; Campbell et al., 1999) or from dietary supplements containing chromium (Anderson et al., 1983; Finley et al., 1996). The efficiency of intestinal absorption of dietary trivalent chromium may or may not be inversely related to trivalent chromium dietary intake. In one study, the efficiency of absorption of 10 μg of chromium daily averaged 2.0% while the efficiency of absorption of 40 μg of chromium daily averaged 0.5%, resulting in a relatively constant daily influx of dietary trivalent chromium into the bloodstream (Anderson and Kozlovsky, 1985). However, in another study, the apparent absorption (estimated from urinary excretion rates) of chromium averaged 0.4% when either 60 μg or 260 μg were consumed daily, resulting in a greater than 3-fold difference in the amount of bioavailable chromium (Anderson et al., 1983).

Within the small low range of absorption efficiencies, the absorption of trivalent chromium following ingestion is strongly influenced by the chemical complex in which the trivalent chromium is administered. Ingestion of trivalent chromium in the form of inorganic salts that are insoluble in water, such as chromic oxide, results in little or no absorption in rats (Gammelgaard et al., 1993) or healthy humans (Finley et al., 1996). By contrast, chromium chloride and trivalent chromium in association with a chelating agent, such as picolinate or nicotinate, are relatively more water soluble and are more efficiently absorbed in the gastrointestinal tracts of rats (Anderson et al., 1997) and humans (Finley et al., 1996). However, chromium chloride and chromium nicotinate are relatively unstable in the presence of starch and may be very poorly absorbed from starch-containing foods, diets or dietary supplements (Anderson et al., 2003). The absorption of trivalent chromium from chromium picolinate has been reported to be greater than that from other forms of chromium (Anderson, 1998).
Studies in humans have shown chromium is excreted mainly in urine, although small amounts are lost in hair, perspiration and bile (Anderson et al., 1983; Davies et al., 1997). The 24-hour urinary excretion rates for normal human subjects consuming 60 μg of chromium daily in food were reported to average about 0.2 to 0.3 μg in unsupplemented males and females and were similar to estimates of the amount of chromium that was absorbed daily (Anderson et al., 1983).

Chromium Status and Aging

Serum chromium concentrations are reported to decrease in 40,872 patients referred by their physicians to an independent medical research clinic and laboratory (Davies et al, 1997). This effect of aging was observed in both males and females and suggests that dietary chromium requirements increase with age in adults.

Influence of chromium on insulin action

Chromium is required by the human body for normal carbohydrate and lipid metabolism (Vincent, 2000, Wilson and Gondy, 1995). It acts as an “insulin-sensitizing agent” (substance that accelerates the clearance of glucose from plasma in the presence of a fixed amount of insulin (Henry, 1998; Mudaliar and Henry, 2001). Chromium is reported to prevent an age-related decline of glucose tolerance in rats. In rats, in the absence of chromium, hypersecretion of insulin is required in order to maintain normal glucose tolerance (Striffler et al., 1999). Various studies in humans involving reduced chromium intake have shown to impair glucose tolerance, which is reversible upon the supplementation of chromium (Anderson et al., 1991; Anderson 1995; Jeejeebhoy et al., 1977). Even chronic low intakes of chromium impair glucose tolerance (Anderson et al., 1991).

Influence of chromium on insulin sensitivity has been shown in the level of cellular actions. Insulin in the extracellular fluid reversibly binds to its receptor within the plasma membrane of a target cell,
triggering movement of vesicle-bound transferrin receptor to the target cell plasma membrane. Cr (III)-bound transferrin, the predominant plasma carrier of Cr (III), binds to the transferrin receptor and is internalized by endocytosis into the cytoplasm of the target cell. Within the cytoplasm, Cr (III) is released from transferrin and binds with apochromodulin, a low molecular weight chromium binding substance, forming the activated messenger, holochromodulin. Activated holochromodulin interacts with the insulin-receptor complex, autoamplifying the occupied receptor's insulin-stimulated tyrosine kinase activity and further increasing tyrosine kinase phosphorylation of the β-subunits of insulin receptors whose α-subunits are occupied by insulin. Phosphorylation of the β-subunit stimulates the transmission of the insulin signal to the cytoplasm with consequent transmembrane glucose transport, increasing intracellular glucose content at the expense of plasma glucose concentration (Anderson, 1998; Vincent, 2000; Vincent, 2000; Evans and Bowman, 1992).

Trivalent Cr (III) ions also inhibit phosphotyrosine phosphatase, an enzyme that dephosphorylates (deactivates) the phosphorylated insulin receptor. Concurrent stimulation of β-subunit phosphorylation and inhibition of its dephosphorylation result in an increased proportion of activated insulin-responsive receptors. Consequently, the greater the proportion of activated insulin receptors responsive to circulating insulin, and the greater the amplification of the insulin signal, the more rapid the resultant decline in plasma glucose concentration and the shorter the duration of pancreatic insulin secretion. When extracellular fluid insulin concentrations subside, insulin releases from its receptor. The unbound receptor has very low affinity for holochromodulin and, in the absence of insulin, releases holochromodulin with Cr (III) into the extracellular fluid (presumably with subsequent diffusion into the

Description of Chromium Picolinate

Chromium picolinate is a stable complex of trivalent chromium (Cr (III)) and picolinic acid. Alternate chemical names for this substance are tris (2-pyridinecarboxylato-N, O$_2$) chromium and chromium (III) trispicolinate (Budavari et al., 1996).

Physical and Chemical Properties of Chromium Picolinate

The physical and chemical properties of chromium picolinate include:
Composition : C - 51.68%, O - 22.95%, Cr -12.43%, N - 10.05%, H - 2.89%
Molecular Weight : 418.31 Daltons
Solubility (in water) : Soluble in water at pH 7 (>100 µg/ml)
Solubility (in chloroform): Soluble in chloroform (2.0 mM)

The Chemical Abstracts Service ("CAS") Registry Number for chromium picolinate is 14639-25-9. The empirical formula for chromium picolinate is C$_{18}$H$_{12}$CrN$_3$O$_6$. The structural formula for this compound is shown in Fig 5 (Budavari et al, 1996).

![Fig 5: The structural formula of chromium picolinate](image-url)
Chromium Literature Review

In Vitro Studies Evaluating the Effects of Chromium Picolinate on Insulin Sensitivity and Glucose Metabolism

Chromium has been shown to enhance target cell sensitivity to insulin by increasing the binding of insulin to its receptor sites, initial receptor site activation and post-receptor transmission of intracellular signaling (Anderson, 1998; Vincent, 2000; Vincent, 2000; Evans and Bowman 1992). Chromium picolinate has been shown to enter target cells and to bind to apochromodulin intact (i.e., as chromium picolinate) (Hepburn and Vincent, 2003) and enhance intracellular signaling by serving as an “auto amplifier” for intracellular insulin signals, such as insulin receptor mediated tyrosine kinase activities (Wang et al., 2002; Wang et al., 2003).

In cultured human skeletal muscle cells, chromium picolinate significantly potentiated insulin-stimulated glucose uptake and glycogen synthesis (Wang et al, 2002; Wang, et al., 2003). In addition, the intracellular activity of insulin receptor substrate-1 (IRS-1) associated phosphotidylinositol 3-kinase (PI3K) activity (a downstream member of the insulin signaling cascade (Anderson, 1998) was significantly increased by chromium picolinate in a concentration-dependent manner (Wang et al., 2002; Wang et al., 2003). Those findings indicate that chromium picolinate acted to increase the sensitivity of human skeletal muscle cells to fixed amounts of insulin.

Chromium picolinate also may enhance insulin-receptor interactions. In cultured rat skeletal muscle cells, in a concentration at which chromium chloride and chromium nicotinate had no effect, chromium picolinate significantly increased insulin-receptor binding and the subsequent uptakes of glucose and leucine (Evans and Bowman, 1992). In addition, the presence of chromium picolinate also significantly increased the fluidity of skeletal muscle cell
membranes, which may have further facilitated insulin-receptor binding (Evans and Bowman, 1992).

**Preclinical Studies Evaluating the Effects of Chromium Picolinate Supplementation**

Dietary supplementation with chromium picolinate has been shown to increase insulin sensitivity and glucose uptake in animals with impaired glucose tolerance. For example, the addition of chromium picolinate to the drinking water of a genetic strain of rat prone to obesity and insulin resistance syndrome (JCR-LA corpulent) for 12 weeks, providing approximately 80 mcg of chromium as chromium picolinate daily per kg of bodyweight, significantly increased insulin-stimulated membrane translocation of the myocyte GLUT4 glucose transporter and the rate of glucose clearance from the blood but significantly decreased fasting plasma insulin concentration, compared to the effects of unsupplemented water (Cefalu et al., 2002). Interestingly, such relatively short-term supplementation with chromium picolinate had no effects on lean rats with normal glucose tolerance; rather than being refractory to chromium picolinate supplementation, rats with normal glucose tolerance may have limited capacity to further stimulate GLUT4 kinetics or glucose uptake in the presence of adequate amounts of insulin.

Similarly, dietary supplementation with chromium picolinate has increased insulin sensitivity and glucose uptake in animals with diabetes. For example, in rats treated with dexamethasone in order to produce glucocorticoid-induced diabetes, concurrent dietary supplementation with chromium picolinate (30 mg/kg/day) prevented the postchallenge hyperinsulinemia and loss of glucose tolerance exhibited by unsupplemented dexamethasone-treated rats (Kim et al., 2002).
Clinical Studies Evaluating the Effects of Chromium Picolinate Supplementation in Individuals with Pre-Diabetes

Cefalu et al (1999) studied the impact of dietary supplementation with chromium picolinate on 29 obese middle-aged human subjects with mild fasting hyperinsulinemia but without hyperglycemia. After both 4 and 8 months of daily dietary supplementation with either 1000 µg of chromium (in the form of chromium picolinate) or placebo, subjects receiving daily dietary supplementation with chromium picolinate exhibited significantly greater (compared to the response to placebo) increases in insulin sensitivity, although (as expected in normoglycemic individuals) plasma glucose concentrations were unaffected. Similarly, young adults with fasting hyperinsulinemia without hyperglycemia experienced significantly greater (compared to the response to placebo) decreases in fasting plasma insulin concentrations after 90 days of daily dietary supplementation with 200 µg of chromium (as chromium picolinate). Those results demonstrate that supplementation of the diet with chromium picolinate can reduce the likelihood of later development of insulin resistance (and therefore of diabetes) in currently healthy individuals at elevated risk of losing glucose tolerance. Furthermore, in a subset of an uncontrolled 5 week trial of daily dietary supplementation with 200 mcg of chromium (as chromium picolinate), adults with impaired glucose tolerance experienced significantly improved glucose tolerance and significantly decreased fasting insulin concentrations (Anderson et al., 1991).

Clinical Studies Evaluating the Effects of Chromium Supplementation on Individuals with Diabetes

Human clinical studies evaluating the effects of chromium picolinate supplementation in subjects with diabetes are reported in Table-2. The results of these studies demonstrate that daily dietary supplementation with 200 µg to 1000 µg of chromium (as chromium
picolinate) consistently improved hyperglycemia and measures of insulin sensitivity (Cefalu et al., 1999; Anderson et al., 1997; Ravina et al., 1999). One trial of subjects with severe diabetes and daily dietary supplementation with only 200 µg of chromium (as chromium picolinate) failed to observe these effects. Nonetheless, it is clear from these studies that daily dietary supplementation with chromium (as chromium picolinate) significantly improves glucose homeostasis and, in so doing, reduces the risks for the development of diseases for which type 2 diabetes is a causative or contributory etiologic factor.

Other chromium complexes also have been evaluated in human clinical studies (Tables 3 and 4) (Wilson and Gondy, 1995; Anderson et al., 1991; Abraham et al., 1992; Carter et al., 1968). Overall, dietary supplementation with chromium chloride, yeast containing chromium or chromium nicotinate has produced either no effects or inconsistent effects on glucose homeostasis, suggesting that those chromium complexes differ functionally from chromium picolinate.
Table 2: Clinical Studies of the Effects of Dietary Supplementation with Chromium Picolinate on Glucose Homeostasis

<table>
<thead>
<tr>
<th>Author and Year</th>
<th>Subject Characteristics</th>
<th>No. of Subjects</th>
<th>Cr (µg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghosh et al., 2002</td>
<td>Type 2 Diabetes Mellitus</td>
<td>50</td>
<td>400</td>
</tr>
<tr>
<td>Anderson et al., 1997</td>
<td>Type 2 Diabetes Mellitus</td>
<td>180</td>
<td>1000</td>
</tr>
<tr>
<td>Lee and Reasner, 1994</td>
<td>Type 2 Diabetes Mellitus</td>
<td>30</td>
<td>200</td>
</tr>
<tr>
<td>Evans et al., 1989</td>
<td>Type 2 Diabetes Mellitus</td>
<td>11</td>
<td>200</td>
</tr>
<tr>
<td>Jovanovic et al., 1999</td>
<td>Gestational Diabetes Mellitus</td>
<td>20</td>
<td>300–800</td>
</tr>
<tr>
<td><strong>Randomized controlled clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Houweling et al., 2003</td>
<td>Type 2 Diabetes Mellitus</td>
<td>52</td>
<td>500, 1000</td>
</tr>
<tr>
<td>Feng et al., 2002</td>
<td>Type 2 Diabetes Mellitus</td>
<td>136</td>
<td>500</td>
</tr>
<tr>
<td><strong>Uncontrolled (“open label”) clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morris et al., 2000</td>
<td>Type 2 Diabetes Mellitus</td>
<td>5</td>
<td>400</td>
</tr>
<tr>
<td>Cheng et al., 1999</td>
<td>Type 2 Diabetes Mellitus</td>
<td>833</td>
<td>500</td>
</tr>
<tr>
<td>Ravina et al., 1995</td>
<td>Type 1 Diabetes Mellitus; Type 2 Diabetes Mellitus</td>
<td>162</td>
<td>200</td>
</tr>
<tr>
<td>Ravina et al., 1999</td>
<td>Steroid-Induced Diabetes Mellitus</td>
<td>44</td>
<td>300–600</td>
</tr>
<tr>
<td><strong>Uncontrolled (“open label”) clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabinovitz et al., 2000</td>
<td>Type 2 Diabetes Mellitus</td>
<td>39</td>
<td>400</td>
</tr>
<tr>
<td>Bahadori et al., 1999</td>
<td>Type 2 Diabetes Mellitus</td>
<td>16</td>
<td>1000</td>
</tr>
</tbody>
</table>
Table 3: Clinical Studies of the Effects of Dietary Supplementation with Chromium Chloride on Glucose Homeostasis

<table>
<thead>
<tr>
<th>Author and Year</th>
<th>Subject Characteristics</th>
<th>No. of Subjects</th>
<th>Cr (μg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chromium Chloride:</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abraham et al., 1992</td>
<td>Type 2 Diabetes Mellitus; Normal Glucose Tolerance</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>Mossop et al., 1983</td>
<td>Type 2 Diabetes Mellitus</td>
<td>26</td>
<td>600</td>
</tr>
<tr>
<td>Uusitupa et al., 1983</td>
<td>Type 2 Diabetes Mellitus</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>Anderson et al., 1983</td>
<td>Impaired Glucose Tolerance; Normal Glucose Tolerance</td>
<td>41</td>
<td>200</td>
</tr>
<tr>
<td>Martinez et al., 1985</td>
<td>Impaired Glucose Tolerance Normal Glucose Tolerance</td>
<td>17</td>
<td>200</td>
</tr>
<tr>
<td>Riales and Albrink, 1981</td>
<td>Normal Glucose Tolerance</td>
<td>23</td>
<td>200</td>
</tr>
<tr>
<td>Urberg and Zamel, 1987</td>
<td>Normal Glucose Tolerance</td>
<td>16</td>
<td>200</td>
</tr>
<tr>
<td><strong>Non-randomized controlled clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chromium Chloride:</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sherman et al., 1968</td>
<td>Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus or Normal Glucose Tolerance</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Anderson et al., 1991</td>
<td>Impaired Glucose Tolerance Normal Glucose Tolerance</td>
<td>8</td>
<td>200</td>
</tr>
<tr>
<td>Potter et al., 1985</td>
<td>Impaired Glucose Tolerance, Elderly Subjects</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>Levine et al., 1968</td>
<td>Impaired Glucose Tolerance, Elderly Subjects</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>Carter et al., 1968</td>
<td>Children with kwashiorkor</td>
<td>9</td>
<td>250</td>
</tr>
</tbody>
</table>
Review of Literature

Uncontrolled ("open label") clinical trials

<table>
<thead>
<tr>
<th>Chromium Chloride:</th>
<th>Type 2 Diabetes Mellitus; Normal Glucose Tolerance</th>
<th>14 10</th>
<th>150 to 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glinsmann and Mertz, 1966</td>
<td>Malnourished Infants</td>
<td>12</td>
<td>250</td>
</tr>
<tr>
<td>Hopkins et al., 1968</td>
<td>Malnourished Children</td>
<td>15</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 4: Clinical Studies of the Effects of Dietary Supplementation with Other Chromium Complexes on Glucose Homeostasis

<table>
<thead>
<tr>
<th>Author and Year</th>
<th>Subject Characteristics</th>
<th>No. of Subjects</th>
<th>Cr (µg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Randomized controlled clinical trials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Yeast containing Chromium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elias et al., 1984</td>
<td>Type 2 Diabetes Mellitus</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Offenbacher and Pi-Sunyer, 1980</td>
<td>Type 2 Diabetes Mellitus; Normal Glucose Tolerance</td>
<td>4 8</td>
<td>10.8</td>
</tr>
<tr>
<td>Uusitupa et al., 1992</td>
<td>Impaired Glucose Tolerance; Elderly subjects</td>
<td>26</td>
<td>160</td>
</tr>
<tr>
<td><strong>Chromium Nicotinate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juang et al., 2000</td>
<td>Impaired Glucose Tolerance</td>
<td>15</td>
<td>800</td>
</tr>
<tr>
<td>Thomas and Gropper, 1996</td>
<td>Type 2 Diabetes Mellitus; Normal Glucose Tolerance</td>
<td>5 14</td>
<td>200</td>
</tr>
<tr>
<td>Wilson and Gondy, 1995</td>
<td>Normal Glucose Tolerance</td>
<td>26</td>
<td>220</td>
</tr>
<tr>
<td>Lefavi et al., 1983</td>
<td>Normal Glucose Tolerance</td>
<td>34</td>
<td>200 or 800</td>
</tr>
</tbody>
</table>
### Non-randomized controlled clinical trials

*Combination of Chromium Chloride and Yeast containing Chromium*

<table>
<thead>
<tr>
<th>Study</th>
<th>Type 2 Diabetes Mellitus; Normal Glucose Tolerance</th>
<th>43</th>
<th>20</th>
<th>150 or 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabinowitz et al., 1983</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang et al., 1989</td>
<td>Normal Glucose Tolerance</td>
<td>30</td>
<td></td>
<td>15 or 50</td>
</tr>
</tbody>
</table>

### Randomized controlled clinical trials

*Combination of Chromium Chloride and Yeast containing Chromium*

<table>
<thead>
<tr>
<th>Study</th>
<th>Type 2 Diabetes Mellitus</th>
<th>78</th>
<th>223</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bahijiri et al., 2000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Uncontrolled ("open label") clinical trials**

*Yeast containing Chromium*

<table>
<thead>
<tr>
<th>Study</th>
<th>Type 2 Diabetes Mellitus</th>
<th>23</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trow et al., 2000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Clinical Studies Evaluating Chromium Picolinate Supplementation in Individuals with Diabetes**

### Randomized controlled clinical trials

Various randomized controlled clinical trials are studied till date on chromium picolinate. Various trials reported till date is briefly discussed below. A randomized controlled crossover study, which compared the effects of daily dietary supplementation with 400 μg chromium (as chromium picolinate) to the effects of placebo in 50 subjects with type 2 diabetes were studied by (Ghosh et al., 2002). The findings of the study showed supplementation of chromium picolinate sensitizes target tissues to insulin (Ghosh et al., 2002).

A parallel randomized controlled clinical trial studied to compare the effects of placebo to the effects of daily dietary supplementation with either 200 μg or 1000 μg of chromium (as chromium picolinate) in 180 subjects with type 2 diabetes was reported by Anderson et al (1997). The study included subjects most of them were receiving oral medications, predominantly sulfonylureas. After 4 months,
compared the effects on the subjects consumed placebo or 200 μg and 1000 μg of chromium daily. The reductions in plasma HbA1c percentages were sufficiently large in the subjects supplemented with 1000 μg of chromium daily that most of those subjects could be considered to have achieved target plasma HbA1c percentages. The findings of the study concluded that supplemental chromium picolinate to sensitize target tissues to insulin and prevent hyperglycemic glycation of hemoglobin (Anderson et al., 1997).

Another trial in 1989 was studied which compared the effects of daily dietary supplementation with 200 μg of chromium (as chromium picolinate) to the effects of placebo in 11 subjects with type 2 diabetes in a randomized, double-blinded, placebo controlled crossover study, with two 42-day treatment periods and an intervening 14-day "washout" period. The changes in mean fasting plasma glucose concentration and HbA1c percentage in the subjects consuming supplemental chromium picolinate were shown to produce statistically significantly different from the changes that were observed during placebo consumption during both arms of the study. The findings of the study support supplemental chromium picolinate to sensitize target tissues to insulin and prevent hyperglycemic glycation of hemoglobin (Evans et al., 1989).

In contrast to the reports of trials mentioned above, the studies of Lee and Reasner (1994) which compared the effects of placebo to the effects of daily dietary supplementation with 200 μg of chromium (as chromium picolinate) in 30 subjects also receiving combination therapies for type 2 diabetes in a 2-month randomized, double-blinded, placebo controlled parallel study, reported no significant difference in mean fasting plasma glucose concentration or plasma HbA1c percentage.

The studies by Jovanovic et al., (1999) compared the effects of placebo to the effects of daily dietary supplementation with chromium picolinate (4 or 8 μg of chromium per kg of bodyweight) in
30 women with gestational diabetes. 8 weeks of dietary supplementation with chromium picolinate had no effect on mean fasting plasma glucose concentrations or mean plasma HbA1c percentages but produced significantly greater decreases in fasting and post-glucose challenge plasma insulin concentrations in both groups consuming chromium picolinate. The findings of the study suggested that dietary supplementation with chromium picolinate may increase maternal tissue insulin sensitivity during gestational diabetes and may reduce the risk of conversion of gestational diabetes to type 2 diabetes.

Houweling et al., (2003) studied influence of daily dietary supplementation with 500 or 1000 μg of chromium (as chromium picolinate) and compared with placebo. It included 52 subjects with type 2 diabetes and plasma HbA1c percentages > 8% despite daily concomitant administration of oral hypoglycemic agents and >50 U of insulin. The findings of that study reported that dietary supplementation with chromium picolinate decreases hyperglycemic glycation of hemoglobin (Houweling et al., 2003).

Feng et al., (2002) examined the effects of daily dietary supplementation with chromium picolinate in subjects with type 2 diabetes on insulin therapy. A total of 104 patients consumed 500 μg of chromium (as chromium picolinate) daily in addition to receiving insulin therapy; 32 patients were treated with insulin alone. The findings of the study also supported that supplemental chromium picolinate to sensitize target tissues to insulin (Feng et al., 2002).

In short most of the trials report a beneficial effects of chromium picolinate on glycemic levels, glycated haemoglobin levels, insulin resistance in diabetics, few studies also report increase in maternal tissue insulin sensitivity during gestational diabetes. However, few studies report no significant effect on glycemic levels on chromium supplementation.
Uncontrolled ("open label") clinical trials

At least four to five studies are reported which evaluated the role of chromium on glycemic levels and its effect on insulin action. Morris et al (2000) evaluated the effects of daily dietary supplementation with 400 µg chromium (as chromium picolinate) in 5 subjects with newly diagnosed type 2 diabetes. Cheng et al (1999) evaluated the effects of daily dietary supplementation with 500 µg chromium (as chromium picolinate) in 833 subjects with type 2 diabetes concurrently treated with oral hypoglycemic medications and/or insulin. The study reported decrease in the incidence of symptoms of diabetes (fatigue, extreme thirst, frequent micturation) by about 90% during chromium picolinate supplementation.

Ravina et al., (1995) evaluated the benefits of daily dietary supplementation with 200 µg of chromium (as chromium picolinate) in 48 subjects with type 1 diabetes and 114 subjects with type 2 diabetes. The findings of the study showed decrease in hyperglycemic glycation of hemoglobin. In the same study, Ravina et al (1995) further evaluated a subset of 55 subjects who were determined to be 'responders' to supplementation with chromium picolinate. And reported that supplemental chromium picolinate prevents or decreases hyperglycemic glycation of hemoglobin.

Later Ravina et al., (1999) evaluated the effects of daily dietary supplementation with 300 to 600 µg of chromium (as chromium picolinate) in a case series of 44 subjects with steroid-induced insulin-resistant diabetes. The results of the study showed supplemental chromium picolinate sensitizes target tissues to insulin, even in the presence of diabetes-induced by glucocorticoids. Taken together the reports of all uncontrolled studies, which included chromium supplementation, showed a significant reduction in glycemic levels by enhanced insulin action and reduction in glycated haemoglobin levels.
Uncontrolled ("open label") clinical trials

Few studies are reported on efficacy of chromium picolinate in uncontrolled clinical trials (Rabinovitz et al., 2000; Bahadori et al., 1999). Rabinovitz et al (2000) evaluated the effects of daily dietary supplementation with 400 µg of chromium (as chromium picolinate) added to a low-sugar diet in 39 subjects with type 2 diabetes. After 3 weeks, significant decreases were observed in mean fasting plasma glucose concentration (21%) and in mean plasma HbA1c percentage (7%). The findings reported supplementation with chromium picolinate to sensitize target tissues to insulin and prevent or decrease hyperglycemic glycation of hemoglobin. Bahadori et al (1999) evaluated the effects of daily dietary supplementation with 1000 µg of chromium (as chromium picolinate) in 16 obese subjects with type 2 diabetes. After 4 months of chromium picolinate supplementation, mean fasting plasma insulin concentration was significantly decreased (by 38%). The study reported a reduction in fasting hyperinsulinemia, which was found to be consistent and hence concluded daily dietary supplementation with chromium (as chromium picolinate) may reduce the risk for the consequences of loss of glucose tolerance.

Clinical Studies on the Effects of Dietary Supplementation with Chromium Picolinate on Body Composition – Data Concerning Glucose Homeostasis

Although much of the clinical research with chromium picolinate in normoglycemic and healthy subjects has focused on the putative impact of this supplement on body composition, some of these studies also examined carbohydrate metabolism. Such studies have examined elderly men and women (Amato et al., 2000), obese women participating in aerobic exercise or resistance training (Grant et al, 1997), obese adults participating in a study of dietary modification (Pasman et al., 1997), college students participating in a program of
aerobic exercise (Boyd et al., 1998), overweight but nondiabetic older men and women participating in a resistance training program (Joseph et al., 1999), middle-aged women participating in a combined weight training and walking program (Volpe et al., 2001), sedentary young adults (Anderson et al., 1991) and collegiate wrestlers in training (Walker et al., 1998). Daily intake of supplemental chromium picolinate is effective in reducing the risk of diabetes. The daily dietary supplementation with 200 μg to 1000 μg of chromium (as chromium picolinate) is reported to be effective in reducing the risk of developing insulin resistance, prediabetes, type 2 diabetes, certain diseases caused by diabetes and certain diseases caused by insulin resistance and in preventing or delaying the progression of prediabetes to diabetes. (Cefalu et al., 1999; Anderson et al., 1991; Kim et al., 2002; Lee and Reasner, 1994; Amato et al., 2000; Boyd et al., 1998; Althuis et al., 2002).

**Safety of Supplemental Chromium Picolinate**

The safety of chromium picolinate has been extensively reviewed by an independent panel of experts and concluded that chromium picolinate to be safe at current or anticipated future levels of intake. The safety review was used to determine that chromium picolinate is Generally Recognized as Safe (GRAS) for addition to specified categories of foods.

**History of Safe Use in Humans**

In a survey of dietary supplement users, 80% stated that they were aware of chromium picolinate, whereas only 8% knew of other forms of chromium. In a 2001 survey of diabetes educators recommend chromium supplementation to their patients. Of the educators who recommended chromium supplementation, 96% specifically recommended chromium picolinate. In a published survey of pharmacists, chromium picolinate was one of the top five most-recommended supplements (Cerulli et al., 1998).
The chromium picolinate has an excellent safety profile with only seven published individual case reports and one published case series of possible adverse reactions (Olefsky, 2001; Zavaroni et al., 1999; Bunner and McGinnis, 1998; Cerulli et al., 1998; Fowler and Jr. Systemic, 2000; Huszonek, 1993; Martin and Fuller, 1998; Wasser et al., 1997; Young et al., 1999).

The seven individual case reports include two case reports of chromium-induced nephrotoxicity, (Cerulli et al., 1998; Wasser et al., 1997) one report of rhabdomyolysis, (Martin and Fuller, 1998) one report of acute generalized exanthematous pustulosis (Young, et al., 1999), one report of systemic contact dermatitis, (Fowler, 2000) one report of cognitive, perceptual and motor changes (Huszonek, 1993) and one report of sporadic hypoglycemia (Bunner, 1998). In the latter case, daily dietary supplementation with 1000 µg of chromium (as chromium picolinate) was effective in minimizing the hyperglycemia of a young man with diabetes in the absence of medications or exogenous insulin; however, 1000 µg of chromium (as chromium picolinate) appeared to be an excessive level of supplementation. In another case report, a young woman with type 1 diabetes successfully reduced her plasma HbA1c percentage from 11.3% to 7.9% with a 3-month course of chromium picolinate supplementation (providing 600 mcg of chromium daily) without hypoglycemic episodes (Fox and Sabovic, 1998). In a series of cases reported to the Georgia Poison Center, nine individuals experienced adverse reactions (chest pain, flushing, dehydration, agitation, dizziness or headache) that could be attributed to the ingestion of 100 to 6000 µg of chromium (as chromium picolinate) (Gorman et al., 1997).

In over 40 human clinical trials in which healthy adults, bodybuilders, overweight individuals, obese individuals, elderly individuals, individuals with hypercholesterolemia, individuals with diabetes and women with gestational diabetes have participated,
amounts of chromium picolinate supplying between 200 μg and 1000 μg of chromium have been consumed daily for as long as 8 months without adverse reactions (Cefalu et al., 1999; Campbell et al., 1999; Hallmark et al., 1996; Clancy et al., 1994; Anderson et al., 1991; Kim et al., 2002; Lee and Reasner, 1994; Amato et al., 2000; Althuis et al., 2002; Anderson et al., 1987; Trent and Thieding, 1995).

Clinical studies evaluating complete blood counts, liver function, renal function, and electrolyte levels have not detected any abnormal changes after chromium picolinate supplementation in people consuming up to 1000 μg chromium (as chromium picolinate) for up to 8 months (Cefalu et al., 1999; Campbell et al., 2003). In another clinical trial, 10 obese participants consumed 400 μg of chromium (as chromium picolinate) daily for 8 weeks without evidence of oxidative DNA damage, suggesting that the intakes typically used for dietary supplementation with chromium picolinate are safe (Kato et al., 1998).

**Animal Studies of Safety**

Studies of chromium picolinate in animals demonstrate a very low toxicity for the compound. Chromium picolinate has been tested in over 30 studies involving over 50,000 animals. No toxicity has been observed in males, females, pregnant females or their offspring. The safety of chromium picolinate has been tested in cats, chickens, cows, dogs, horses, lambs, pigs, mice and rats. For example, chromium picolinate administration to rats in daily amounts providing up to 15 mg of chromium per kg of body weight for 24 weeks (equivalent to about 5,000 times the human dose of 200 mcg of chromium a day for approximately 20 years) produced no apparent adverse effects (Anderson et al., 1997). There were no negative effects on body weight, organ weights, and plasma or serum concentrations of glucose, cholesterol, triglycerides, or protein, blood
urea nitrogen concentration, plasma lactate dehydrogenase activity or serum enzyme activities. Furthermore, no abnormal changes were observed in the liver or kidneys.

The potential effects of chromium picolinate on reproductive physiology and fertility were examined in swine (metabolically similar to humans). In several studies, daily dietary supplementation of the diets of sows with 200 ppb to 1000 ppb of chromium (as chromium picolinate) produced no negative effects and resulted in significantly greater litter size, higher birth weights, and better piglet and sow health compared to the growth rates and fertility of unsupplemented animals (Lindemann et al., 1995; Lindemann et al., 2002).

Several studies in rats have examined the potential mutagenicity and carcinogenicity of chromium picolinate. Rats intubated with a single bolus of chromium picolinate providing 4,000 to 250,000 mcg of chromium per kg of bodyweight exhibited no increases in chromosome damage (Esber et al., 1999). Similarly, a study that examined the effects of chromium picolinate supplementation on the bone marrow cells of rats found no indication of chromium-picolinate-induced chromosomal damage (Greenberg et al., 1999). The only animal study to suggest that chromium picolinate could produce oxidative DNA damage administered daily intravenous injections of chromium picolinate for 2 months in amounts about 20-fold greater than the typical amounts consumed by humans supplementing their diets (Hepburn and Vincent, 2002). Because such intravenous injection of chromium picolinate results in circulating chromium concentrations several orders of magnitude greater than those resulting from oral ingestion of chromium picolinate, that study is of little relevance to the evaluation of the safety of dietary supplementation with chromium picolinate.
In Vitro Studies of Safety

Under certain non-physiologic conditions in in-vitro studies, chromium picolinate has been reported to cause toxicity. These studies have used extremely high concentrations of chromium picolinate or did not use preparations that were manufactured under Good Manufacturing Practices (GMPs) and contained toxic impurities.

One study observed that the consumption of chromium picolinate in amounts providing chromium in amounts approximately equivalent to the dietary intake of unsupplemented humans produced significantly increased rates of lethal mutations and female sterility in *Drosophilae melanogaster* (fruit flies) (Hepburn et al., 2003). In another study, adding chromium picolinate to the culture medium triggered chromosomal changes in Chinese hamster ovary cells (Stearns et al., 1995). However, the lowest concentration of chromium picolinate found to cause damage in the cell cultures was 3,000 times the concentration measured in the blood of humans after consuming 200 mcg of chromium (as chromium picolinate) daily for 2 months (Rabinovitz et al., 2000). In contrast, exposure of human macrophages in cell culture to supraphysiologic concentrations of chromium picolinate produced only modest increases (considered by the investigators to be inconsequential) in indices of oxidative stress and DNA fragmentation (Bagchi et al., 1997).

Chromium Picolinate GRAS Determination

In April 2000, a panel of experts qualified by scientific training and experience to evaluate the safety of substances added to food released their review of the totality of the available data concerning the safety of chromium picolinate. These data included the results of laboratory, animal, and human studies as well as case reports of possible adverse reactions that might be attributable to chromium
picolinate. The panel concluded that the weight of the evidence clearly demonstrates the safety of chromium picolinate.

In summary, there is significant scientific agreement in support of the following health claims chromium picolinate may reduce the risk of insulin resistance. Chromium picolinate may reduce the risk of cardiovascular disease when caused by insulin resistance. The Chromium picolinate may reduce the risk of abnormally elevated blood sugar levels. Chromium picolinate may reduce the risk of cardiovascular disease when caused by abnormally elevated blood sugar levels. Chromium picolinate may reduce the risk of type 2 diabetes. Chromium picolinate may reduce the risk of cardiovascular disease when caused by type 2 diabetes. Chromium picolinate may reduce the risk of retinopathy when caused by abnormally high blood sugar levels. Chromium picolinate may reduce the risk of kidney disease when caused by abnormally high blood sugar levels.

3.2.2 Vanadium

The most straightforward experiments to interpret concerning the effects of oxovanadium compounds on biological systems are carried out in cell-free systems. In these systems cellular interconversion of oxovanadium compounds in not as serious a problem as it is in whole cell experiments.

Inhibition

Vanadium is known to inhibit various enzymes like Na\textsuperscript{+}K\textsuperscript{+}ATPase, Ca\textsuperscript{2+} Mg\textsuperscript{2+} ATPase, K\textsuperscript{+} H\textsuperscript{+} ATPase, DYNEIN ATPase.

Na\textsuperscript{+} K\textsuperscript{+} ATPase

Vanadium has been known since 1965 to inhibit Na\textsuperscript{+} K\textsuperscript{+} ATPase (Rifkin, 1965). However, it was shown only recently by three independent laboratories that certain vanadium compounds are among the most potent known inhibitors of this enzyme system (Cantley et al., 1977; Nechay and Saunders, 1978). The inhibitory
effects of vanadium on the Na\textsuperscript{+} K\textsuperscript{+} ATPase in vitro first reawakened interest in interactions of vanadium with metabolic processes. The mechanism of vanadate inhibition for most of the enzyme systems is well understood for enzymes having a phosphoprotein intermediate in their catalytic cycle. These enzymes appear to have a phosphate linked to the protein through an aspartate residue. The vanadate, which has trigonal bipyramidal geometry, binds to the active site of the enzyme and acts as a transition state analogue. However, it is not clear that inhibition of the Na\textsuperscript{+} K\textsuperscript{+} ATPase by vanadyl (IV) (North and Post, 1980) is much weaker than that found by vanadate (V).

\textit{Ca}\textsuperscript{2+} Mg\textsuperscript{2+} ATPase

Studies have been done mainly on enzymes from sarcoplasmic reticulum of mammalian skeletal muscle and heart and from human erythrocytes. The red cell Ca\textsuperscript{2+} Mg\textsuperscript{2+} ATPases is several times more resistant to VO\textsubscript{3}\textsuperscript{-} than Na\textsuperscript{+} K\textsuperscript{+} ATPase (Bond and Hudgins, 1980). Ca\textsuperscript{2+} Mg\textsuperscript{2+} ATPase of sarcoplasmic reticulum requires at least 10 times higher concentration of VO\textsubscript{3}\textsuperscript{-} for 50\% inhibition than that of erythrocytes (Wang et al., 1979; Hangenmeyer et al.; 1980; Pick, 1982).

In reconstituted ghosts, intracellular VO\textsubscript{3}\textsuperscript{-} (5 X 10\textsuperscript{-5} M) inhibits active Ca\textsuperscript{2+} efflux; this inhibition is promoted by intracellular Mg\textsuperscript{2+} and K\textsuperscript{+} and is antagonized by extra cellular Ca\textsuperscript{2+} (Rossi et al., 1981). The sensitivity of Ca\textsuperscript{2+} pump to VO\textsubscript{3}\textsuperscript{-} in vesicles made purified red cell Ca\textsuperscript{2+} Mg\textsuperscript{2+} ATPase is similar to that observed in whole erythrocytes ghosts (Niggli et al., 1981). In the intact red cell, external VO\textsubscript{3}\textsuperscript{-} (5 X 10\textsuperscript{-5} M) does not inhibit the Ca\textsuperscript{2+} pump (Szasz et al., 1981). When exposed to 5 X 10\textsuperscript{-4} M VO\textsubscript{3}\textsuperscript{-}, fresh erythrocytes become highly labeled with externally added Ca\textsuperscript{2+}, which suggests some penetration of VO\textsubscript{3}\textsuperscript{-} into the cells as well as inhibition of the outwardly directed Ca\textsuperscript{2+} pumping ATPase (Varecka and Carafoli, 1982). The VO\textsubscript{3}\textsuperscript{-} induced accumulation of Ca\textsuperscript{2+} by red cells cause a massive efflux of K\textsuperscript{+},
suggesting either an activation of the Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel in the erythrocyte membrane (Varecka and Carafoli, 1982) or that the intracellular VO\textsuperscript{2+} metabolite, similarly to Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and Pb\textsuperscript{2+} can open the K\textsuperscript{+} channel (Siemon et al., 1982).

\textit{K\textsuperscript{+} H\textsuperscript{+} ATPase}

VO\textsubscript{3}\textsuperscript{-} inhibits microsomal gastric mucosa K\textsuperscript{+} H\textsuperscript{+} ATPase, which is an expression of a part the gastric H\textsuperscript{+} pump; proton transport by gastric microsomal vesicles and acid secretion by gastric glands are also reduced (O'Neal et al., 1979; Faller et al., 1981; Faller et al., 1982). VO\textsubscript{3}\textsuperscript{-} also inhibits urinary acidification by the turtle bladder; the mechanism has not been determined (Arruda et al., 1981). A bacterial membrane-bound proton-translocating ATPase was found to be sensitive to VO\textsubscript{3}\textsuperscript{-}.

\textit{Dynein ATPase}

Dynein is the collective name for either Ca\textsuperscript{2+}- or Mg\textsuperscript{2+}- requiring high molecular weight ATPase associated with microtubules. They function in the transduction of the chemical energy provide by ATP hydrolysis into mechanical work such as ciliary and flagellar motility and may have roles in chromosome movement, explasmic transport, and the intracellular movement of membrane-bound vesicles (Gibbons, 1982; Johnson and Porter, 1982). The sources of dyneins studied in detail have been the flagella and cilia of the \textit{Tetrahymena} and the sea urchin.

VO\textsubscript{3}\textsuperscript{-} at concentrations on the order of 10\textsuperscript{-6}–10\textsuperscript{-7} irreversibly inhibits both the isolated dynein ATPase activity and the motility of demembranated sea urchin of porcine sperm flagella and sea urchin embryo cilia (Gibbons et al., 1978; Kobayashi et al., 1978a). Mg\textsuperscript{2+}-activated dynein system is over 30 times more sensitive to VO\textsubscript{3}\textsuperscript{-} than the Ca\textsuperscript{2+} - activated one (Gibbons et al., 1978; Shimizu, 1982). The inhibition of Mg\textsuperscript{2+}-stimulated enzyme activity is noncompetitive with ATP (Gibbons et al., 1978; Shimizu, 1981; Anderson and Purich, 1981).
1982), as is the reduction of flagellar beat frequency by VO$_3^-$ (Gibbons et al., 1978). The intact sea urchin spermatozoa are not inactivated by $10^{-2}$M VO$_3^-$, and those of the pig require $10^{-3}$ M VO$_3^-$ for complete paralysis (Kobayashi et al., 1978a). Other observations extend and amplify these findings (Majumdar, 1981; Rikmenspoel et al., 1981; Satir et al., 1981; Goodenough and Heuser, 1982; Penningroth, 1982; Penningroth et al., 1982; Warner and McIlvain, 1982).

Myosin and actinomyosin ATPase are not inhibited by VO$_3^-$ concentrations below $5 \times 10^{-4}$ M (Cantely et al., 1977; Gibbons et al., 1978). Other studies, however, have demonstrated an irreversible, slow-onset inhibition of myosin and actomyosin ATPases by millimolar concentrations of VO$_3^-$, the mechanism is the formation of a stable myosin-ADP-vanadium complex (Goodno, 1979; Goodno and Taylor, 1982; Kawamura and Tawada, 1982). The difference in kinetics and sensitivity to VO$_3^-$ offers an opportunity for distinguishing between the actions of dynein and myosin in different forms of cell motility (Gibbons et al., 1978; Yamin and Tamm, 1982).

VO$_3^-$ at $\sim 10^{-5}$ M has been shown to inhibit mitotic spindle in lysed cells (Cande and Wolniak, 1978) and translocation of pigment granules in permeabilized erythrophores (Stearns and Ochs, 1982) or when injected into the cell (Beckerle and Porter, 1982).

**Stimulation**

The mechanism by which vanadate stimulates biological processes is much less well understood than the mechanism of inhibition by vanadate. One problem has been the lack of data demonstrating which oxovanadium form of vanadate is the active species. At the current time the chemical mechanisms of stimulatory actions of oxovanadium compounds remain elusive.
Adenyl Cyclase

This enzyme is one of the firsts to have been shown to be stimulated by vanadate in the +5 oxidation state. Cyclic AMP is formed by catalytic action of adenyl cyclase and is inactivated by phosphodiesterase. VO$_3^-$ (>10-5 M), along with fluoride, catecholamines, vasopressin, prostaglandin E1, parathyroid hormone, and glucagons, stimulates isolated adenyl cyclase activity from a variety of sources (Grupp et al., 1979; Hackbarth et al., 1980; Krawietz et al., 1980; Pertseva et al., 1982; Schmitz et al., 1980). The action of VO$_3^-$ is not shared by V$^{4+}$ and V$^{3+}$ compounds (Schmitz et al., 1982). It is independent of hormones and inhibition of phosphodiesterases by theophylline (Schwabe et al., 1979) and differs from that of fluoride (Lichstein et al., 1982; Johnson, 1982; Krawietz et al., 1982). The postulated mechanism involves formation of an enzyme complex with VO$_3^-$ via guanine nucleotide regulatory protein (Krawietz et al., 1982). Concentrations of VO$_3^-$ in excess of 5mM inhibit adenyl cyclase (Lichtstein et al., 1982).

Vanadium-Dependent NADH oxidation

Vanadate-stimulates NADH oxidation activity in the absence of added protein. Stimulation by protein of vanadate-dependent NADH oxidation by plasma membranes was reported by Ramasarma et al. (1981). This plasma membrane activity has been studied in plants (Briskin et al., 1985), the yeast S. Cerevisiae, cardiac cells (Erdman et al., 1979), erythrocytes (Vijaya et al., 1984) and rat liver (Coulombe et al., 1987). The plasma membrane-mediated, vanadate stimulated by paraquat, a superoxide dismutase, a superoxide radical scavenger, and is stridulated by paraquat, a superoxide producer. The reaction does not occur anaerobically.

The purpose of plasma membrane-stimulated vanadate-requiring NADH oxidation activity remains elusive. The function of the membrane protein of the reaction appears to be the production of
superoxide, which is potentially toxic to the cell, while the continuation of the chain causes the production of hydrogen peroxide, another toxic compound. It has been suggested that the reaction is involved in the generation of heat and/or vanadium toxicity. However, it has been shown that the facultative anaerobic yeast, *S. Cerevisiae*, is equally sensitive to vanadate when grown aerobically or anaerobically, which implies that no oxidative processes of any type are involved in vanadium toxicity.

**Protein Phosphorylation**

The effect of vanadium compounds is not well understood and only a small fraction of the experiments in this field have been done in cell free systems. In isolated cell membranes from A-431 cells it has been reported that μmolar concentration of vanadate inhibit the membrane-dependent dephosphorylation of histones containing phosphotyrosine, but not those containing phosphoserine or phosphothreonine (Swarup et al., 1982). Vanadate (in μmolar quantities) also increased tyrosine phosphorylation in a membrane fraction from a human lymphoblastoic cell (Earp et al., 1983). In experiments using insulin-receptor enriched fractions obtained from microsomal membranes of human placenta and rat liver, vanadate activation of the autophosphorylation of the insulin receptor was observed. The autophosphorylation occurred in a dose dependent manner, showing a half-maximal response at 30μM (Gherzi et al., 1988). In experiments using cellular fractions from rat brain cortex vanadate and vanadyl ions stimulated total phosphorylation of proteins from synaptic membranes and to a lesser extent from mitochondria (Krivanek, 1988). Vanadate has been shown to rapidly esterify the hydroxyl groups of aromatic rings to yield a phenyl vanadate. The vanadate esterification proceeds with an equilibrium constant much larger than that of the corresponding phosphate esterification reactions (Tracy and Grasser, 1986).
Organs affected by exogenous vanadium

Exogenously added oxovanadium compounds have been shown to have many physiological effects on various organ systems. At the current time it is not possible to differentiate primary from secondary effects of vanadium. In addition the form of oxovanadium which is affecting each system has not yet been determined due to the pH, concentration and oxidation-reduction changes that can occur as the oxovanadium compounds move through the body. Throughout the body the most consistent response is one of vasoconstriction. There have been some studies concerning the effect of vanadate on eye, ear, liver and brain (Nechay, 1984; Nechay et al., 1986). However, the bulk of the work consists of studies utilizing the kidney and the heart.

Heart

Since inhibition of Na+K+ ATPase has been implicated in the positive inotropic action of digitalis, there is much interest in the cardiac effect of VO$_3^-$.

The addition of oxovanadium compounds to cardiac muscle has been shown to have both positive and negative inotropic effects depending upon the animal species studied and the amount of drug used in the study (Inciarte et al., 1980; Takeda et al., 1980). Alterations in the cardiac function by vanadate have not been linked to changes in K$^+$ transport in the heart implying that interaction with Na$^+$K$^+$ATPase is not the primary action of oxovanadium in the heart. VO$_3^-$ stimulates adenyl cyclase and so can increase the concentration of cyclic AMP in cardiac muscle; this effect also seems to be unrelated to its inotropic actions. VO$_3^-$ increases the force of contraction of isolated rat atrial muscle by increasing the Mn$^{2+}$ sensitive superficial Ca$^{2+}$ pool, which is related to the beat-to-beat control of force of contraction, on the other hand, VO$_3^-$ lowers the force of contraction in guinea pig atrial muscle by inhibiting slow Ca$^{2+}$ channels (Stemmer et al., 1983). Compounds of vanadium in +4
and +3 oxidation states do not share with VO$_3^-$ the positive isotropic effect on isolated cat papillary muscles and stimulation of adenyl cyclase (Schmitz et al., 1982).

High concentrations of VO$_3^-$ (>10$^{-4}$ M) may inhibit (like ouabain) or stimulate (like insulin) the uptake of K$^+$ by heart muscle cells from various species; both types of effect may be associated with the positive inotropic effect (Werdan et al., 1982). It was shown previously for other tissues that VO$_3^-$ the stimulating effect of insulin on glucose oxidation (Degani et al., 1981) and transport, which appears to be associated with or mediated by rise in cytoplasmic Ca$^{2+}$ concentration (Clausen et al., 1981). Another suggestion is that the stimulation by VO$_3^-$ of rat heart protein kinase C, which promotes the phosphorylation of the membranes of the sarcoplasmic reticulum, may play a role in strengthening myocardial contraction by increasing sarcoplasmic reticulum stores of Ca$^{2+}$ (Catalan, 1982).

The addition of calcium channel blockers, such as verapamil, attenuate increases in pressure and diminish increases in cardiac output caused by vanadate (Herzig et al., 1981).

**Kidney**

Renal effects of vanadium include a mixture of haemodynamic and parenchymal actions and, like cardiac effects, are characterized by unexplained profound species differences. Vanadate accumulates to greatest levels in the kidneys and has a strong consistent vasoconstriction effect in that organ (Nechay, 1984). Vanadate causes strong natriuresis in various animals. It produces large diuresis in the rat but not in dog (Inciarte et al., 1980; Lopez-Novoa et al., 1982a, 1982b) and cat (Larsen and Thomson, 1980a, 1980b). Vasoconstriction, lowering of renal blood flow and glomerular filtration rate (GFR) are prominent. The vasoconstriction produced in rat (Day et al., 1980) is much less intense than that in the more sensitive dog. In the rat, GFR may rise simultaneously with
increased renal peripheral resistance, suggesting a post capillary vasoconstrictor effect (Kumar and Corder, 1980), in the dog and cat only a fall in GFR was seen. The effect of vanadate and other diuretics are additive. In isolated systems, vanadate is more potent when present in lumen compared to the peritubular bath of isolated perfused renal tubules (Day et al., 1980). Vanadate also inhibits transtubular secretion of p-aminohyppuric acid (Edwards and Grantham, 1983) and inhibits the action of vasopressin in the collecting tubule (Steffen et al., 1981). The possible inhibitory interactions of vanadate on Na⁺K⁺ATPase have been considered as a mechanism of action of vanadate in kidney, since K⁺ and Na⁺ fluxes are affected by vanadate (Higashino et al., 1983; Churchill and Churchill, 1980). Vanadate also reduces renal rennin secretion in rat kidney slices (Churchill and Churchill, 1980).

**Eye and Ear**

Topical application of VO₃⁻ lowers intraocular pressure in the rabbit and monkey, presumably by inhibition of Na⁺K⁺ATPase in the ciliary body and consequently reduction of intraocular by inhibition of Na⁺K⁺ATPase in the ciliary body and consequently reduction of intraocular fluid formation (Becker, 1980; Krupin et al., 1980). It proves only marginally effective in human ocular hypertension (Krupin et al., 1980; Krupin et al., 1983). VO₃⁻ (>10⁻⁴ M) also inhibits active Na⁺ and Cl⁻ transport in the isolated frog cornea (Candia and Podos, 1981). An ATPase from toad retinal rod outer segments that may have a dynein function involved in light-controlled structural changes is sensitive to VO₃⁻ (Thacher, 1981).

Ototoxicity of locally applied VO₃⁻ was studied in guinea pigs. Both the endocochlear and microphonic pritentials are inhibited by oubain. Although VO₃⁻ inhibits Na⁺K⁺ATPase of stria vascularis in vitro, it causes an increase in the cochlear potential followed by a gradual decrease and depression of microphonic potential when applied
perilymphatically. These results were interpreted to suggest that VO$_3^-$ acts by depolarizing the hair cells of the organ of Corti (Marcus et al., 1981, 1982).

**Brain**

Whole brain microsomal Na$^+$/K$^+$ ATPase is several times less sensitive to VO$_3^-$ than is the kidney or heart enzyme in four mammalian species (Nechay and Saunders, 1978), it is not known whether it is related to the presence of two types of Na$^+$/K$^+$ ATPase in the brain (Sweadner, 1979) or to other factors. Consistent with inhibition of Na$^+$/K$^+$ ATPase, VO$_3^-$ interferes with the uptake of $^3$H-norepinephrine by rat cerebral cortex slices; the high concentrations ($<10^{-4}$ M) required suggest poor intracellular penetration of VO$_3^-$. Vanadium intoxication in rats causes changes in brain catecholamine levels (Witkowska and Brzezinski, 1979). VO$_3^-$ (10$^{-3}$ M) also diminishes muscarinic binding sites in homogenates of rat corpus striatum (Danielsson et al., 1983).

The signs of vanadium toxicity in man include tremor and central nervous system depression (Done, 1979). It has been suggested that manic-depressive disorders may be associated with increased vanadium levels and a genetically defective Na$^+$ pump hypersensitive to inhibitors (Naylor and Smith, 1981a, 1981b; Naylor, 1983). Plasma vanadium concentrations in manic-depressed subjects were twice that of normal controls and declined after recovery (Dick et al., 1982). Encouraging results were obtained in the therapy of manic depressive psychosis with a low vanadium diet and therapy with EDTA or reducing agent, ascorbic acid, and methylene blue (Narsapur and Naylor, 1983). Antidepressants such as imipramine and indalprine may also reduce VO$_3^-$ to VO$^{2+}$. Other investigators extended these observations to several ATPase in erythrocytes of patients with effective disorders and found the best correlation between mood and Ca$^{2+}$/ATPase activity (MacDonald et al., 1982).
Physiological Effects Of Vanadium

Effects of Vanadium on cellular processes

Exogenous vanadate addition has been shown to alter various cellular processes. Vanadate inhibited the ATP-dependent degradation of proteins in reticulocytes (Tanaka et al., 1984). Vanadate has also been shown to affect lipid metabolism. Administration of vanadate intraperitoneally to rats has been shown to increase brain cholesterol levels and the ratio of cholesterol/phospholipid. Vanadate addition had a similar, though reduced, effect on lipids in in-vivo experiments with cultured cells (Catalan, 1987). Vanadate has also been reported to inhibit the rate limiting step in cholesterol biosynthesis, the synthesis of mevalonate, which is catalysed by HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase (Menon et al., 1980).

Cell proliferation, Differentiation and Phosphorylation

The stimulatory actions of vanadium on protein phosphorylation may be related to its hormone-mimetic activities, its potential role in the regulation of cell proliferation and differentiation and its anticarcinogenic properties.

Cell Proliferation and Differentiation

Vanadate addition to the culture medium has produced numerous stimulatory effects on cell proliferation and differentiation. Quiescent human fibroblasts treated with 4μm vanadate were stimulated to divide and DNA synthesis increased. However, at 210mM vanadate in the culture medium no stimulation of DNA synthesis was found (Carpenter, 1981). Vanadate was also found to replace interleukin 3 as a growth factor in a mouse cell line (Tojo et al., 1987). In friend erythroleukemia cells vanadate blocked differentiation without affecting cell viability (English et al., 1983). In human lymphocytes vanadate had an inhibitory effect during the first three days of cell
culture, when both differentiation and proliferation take place, but in enhanced DNA synthesis, acting as a co-mitogen in subsequent days of culture (Marini et al., 1987), vanadate added earlier to the culture medium had been found to be mitogenic for a subpopulation of thymus cells, but not for splenocytes and vanadate has been shown to stimulate bone cell proliferation and collagen synthesis at 5-15 mM (Lau et al., 1988). Vanadate, in vitro, potentiated the estrogen stimulation of proliferation of a mouse laying cell line (Sala et al., 1987).

**Phosphorylation / Dephosphorylation**

The effects of vanadate treatment of phosphoprotein levels have been examined in various systems. A stimulatory effect of vanadate treatment on protein tyrosine phosphorylation in fibroblasts has been reported (Klarlund et al., 1988). Biochemical assays for phosphotyrosine residues were used to show that vanadate treatment stimulated phosphotyrosine production in mouse lens cell (Gentleman et al., 1987). The occurrence of protein phosphorylation has been studied in conjunction with the insulin like activity of vanadate. Phosphorylation of insulin receptor in vanadate treated adipocytes (Tamura et al., 1984a) was reported. However, other workers using an antiphosphotyrosine antibody were unable to see an increase in tyrosine protein phosphorylation in the adipocyte system (Mooney et al., 1989). Most recently the effect on oncogenes on incubation with vanadate has been reported. Vanadate stimulated phosphorylation of \( fms \) (Tamura et al., 1986) and \( src \) (Ryder and Gordon, 1987; Collett and Belzer, 1987) proteins. Those oncogenes are believed to be phosphorylated by protein kinase C whose stimulation by the degradation of membrane phosphoinositol depends on intracellular calcium mobilization. Vanadate has also been shown to induce inositol phosphate formation and inhibit inositol phosphate degradation in CCL39 cell lines (Paris and Poussegur, 1987).
**Cellular and Intracellular Motility**

The motility of sperm, cilia and chromosomes have been reported to be inhibited by vanadate (Ramasarma and Crane, 1981). This effect may be related to the inhibition of dynein and muscle ATPases by vanadate (Nechay, 1984; Nechay et al., 1986). Intracellular movement of vesicles may be stimulated by vanadate. It has been demonstrated that vanadate addition to yeast growth medium stimulated the accumulation of the fluorescent dye Lucifer yellow in yeast, which is used as a measure of endocytosis.

**Transport across the plasma membrane**

The effects of vanadate-treatment on transport into cells have been extensively studied in a few cell types. Vanadate-treated rat adipocytes have been used to study transport in many experiments designed to characterize the insulin-mimetic activity of vanadate. The effect of vanadate treatment on glucose and cation transport in isolated adipose tissue and skeletal muscle has been studied (Clausen et al., 1981). In fat pads 0.5 to 5 mM vanadate stimulated glucose uptake and efflux of 3-O methylglucose and increased calcium wash out from preloaded fat pads. In muscle 3-O methylglucose efflux and calcium wash out were stimulated, while in other muscle cells vanadate treatment increased K⁺ while decreasing Na⁺. The stimulatory effect of vanadate on glucose transport in this case appears to be associated with or mediated by rise in cytoplasmic calcium level.

Vanadate has been shown to stimulate ion movement across various portions of the gastrointestinal tract. In rabbit colonic epithelia μM amounts of vanadate on the serosal side caused the stimulation of chloride secretion, without affecting sodium transport. In rat jejunum vanadate appears to have a concentration dependent affect on transport. At 0.1 mM it increased influx and efflux of alanine across the mucosal border, but at higher concentrations, (1 to...
10mM) it decreased mucosal to serosal flux and influx of alanine concurrently with reducing Na⁺ K⁺ ATPase activity in the basolateral membranes (Hajjar et al., 1987). It has been suggested that a low concentration effect result from vanadyl stimulation of adenyl cyclase.

Vanadate affects on transport in red blood cells have also been monitored. Calcium transport into red cells was stimulated by 0.5mM vanadate, accompanied by a large efflux of K⁺. It is hypothesized that in these cells vanadate moves inside the cells and the increased calcium accumulation is due to decreased activity of the Ca²⁺ ATPase which pumps calcium out of the cell (Varecka and Carafoli, 1982).

**Intracellular Ca²⁺ Movement**

Vanadate interference with intracellular Ca²⁺ movement would link the hormonal affects of vanadate to interactions of protein kinase C. ATP dependent calcium uptake by microsomal fractions of rat salivary gland was inhibited by vanadate (Kangasuntheran and Theo, 1982). Alterations in calcium movement have been associated with the inotropic effects of vanadate (Akera et al., 1983). The increased vanadate induced force of contraction of isolated rat atrial muscle is associated with increase of Mn sensitive calcium pool, while vanadate lowering of the force of contraction of guinea pig atrial muscle has been associated with inhibition of the closing of calcium channels, vanadate stimulation of glucose metabolism in adipocytes and skeletal muscle is associated with a rise in cytoplasmic concentration (Erdmann, 1980).

**Oxidation-Reduction Processes**

From vanadate chemistry it is expected that vanadium ions would affect oxidation-reduction processes in cells. However, there has not been extensive work directed at identifying redox processes in cells that may be affected by vanadate. Whether vanadate dependent
NADH oxidation activity is related to any cellular phenomena is not known. The ability of *S. Cerevisiae* to reduce vanadate to vanadyl has been shown to be increased in vanadate resistant mutants, implying that this pathway may aid in lessening the toxic effects of vanadate (Willisky et al., 1985). Vanadate has been taken up by cells and rapidly reduced to vanadyl (Degani, 1981; Willsky et al., 1984). This reduction is mediated by glutathione and cellular catechols (Macara et al., 1980; Cantley and Aisen, 1979). The formation of an intermediate vanadate-thioester has been proposed as a part of a preequilibrium step in glutathione reduction of vanadate to vanadyl (Legrum, 1986). Administration of vanadate to mice depresses the oxidation rate of formation to CO$_2$. While *in vitro* in cytosolic liver fractions vanadate inhibited the enzymatic transfer of folate to tetrahydrofolic acid (Bruch et al., 1987). In mice vanadate inhibited the oxidation demethylation of substrate of the cytochrome P-450 dependent monoxygenase system. The effect was reduced by pretreatment of the animals with ascorbic acid and vanadyl sulphate did not produce as marked an effect as orthovanadate (Heide et al., 1983). It appears that in this system vanadate is the more potent inhibitor and reduction to vanadyl by ascorbate lessens the effect of vanadate. Effects of vanadate on one carbon metabolism and cytochrome P-450 dependent processes certainly implicate vanadate as a modulator of oxidation-reduction reactions in the cell.

*Toxicological Effects*

Until recently interest in vanadium was confined primarily to its toxicological properties in certain species. In humans, the major side effects of vanadate are eczema, dermatitis, conjunctivitis, irritation of upper and lower respiratory tract, the later resulting into atrophic rhinitis, pharyngitis, chronic bronchitis and diffuse pulmonary fibrosis. Central nervous system manifestations are characterized by tremors, headaches, tinnitus and changes in mental status.
Vanadium is toxic after acute parenteral administration, but much less so after oral administration because it is only poorly absorbed by the gastrointestinal tract (Llobet and Domingo, 1984). In diabetic animals chronically treated with oral vanadium, the serum concentration of the element averages 20μM (Brichard et al., 1991), which is about 1000-fold greater than in untreated controls. Even higher concentrations may be reached in some tissues (for example, bone, and kidney), in which vanadium accumulates (Dai et al., 1994). The most obvious side effect of vanadium treatment is the decrease in body weight gain. Diarrhoea can occur but can be resolved with tapering dosage. Although there are exceptions, long-term (up to one year) oral administration of vanadyl to diabetic rats has usually been found not to affect haematological indices and not to alter hepatic or renal function, as shown by morphology and by the lack of increases in liver cytolytic enzymes or in creatinine (Dai et al., 1994). Despite the known contractile effects of vanadium in smooth muscle, oral treatment with the element did not increase blood pressure of normal or diabetic rats. In contrast, vanadium was even reported to lower both blood pressure and elevated plasma insulin levels of spontaneously hypertensive rats (Bhanot et al., 1994).

Little is know about the toxic systemic effects of vanadium in humans. Most investigators have been concerned by occupational exposure to the element, which may result in toxicity by the respiratory route (Goldfine et al., 1994). From the few controlled studies or therapeutic trials in which vanadium was given orally, it emerges that the most common side effect is a mild gastrointestinal intolerance (International Programme on Chemical Safety, 1988; Goldfine et al., 1994; Cohen et al., 1995), no marked perturbations of biochemical parameters were detected on the screening laboratory profiles (Dimond et al., 1963, Goldfine et al., 1994; Cohen et al., 1995). Although the doses of vanadium were substantially lower
than in animal studies, the concentrations reached in plasma (1-5μM) were only five to twenty fold lower, i.e., two orders of magnitude higher than in untreated subjects. However, these studies were limited in time. The long term repercussions of a marked elevation of plasma vanadium levels in humans remain unknown. In this respect, the effects of vanadium on tyrosine kinases, which are involved in cell growth and differentiation, deserve particular attention because they raise the problem of the potential carcinogenicity of vanadium. Transformation of different cell lines can be induced by vanadate (Klarlund, 1985). However, no increase in the incidence of cancer was observed in the few studies, which addressed this question in whole animals.

Since vanadyl compounds seem to be less toxic than vanadate when given acutely (Llobet and Domingo, 1984) and could be the active form in target tissues, the use of the reduced form has been suggested for therapeutic purposes (Pederson et al., 1989; Brichard et al., 1991). However, a recent study failed to demonstrate any advantage of vanadyl over vanadate salts, with respect to tolerance and efficacy in diabetic rats (Becker et al., 1994). Nevertheless, attempts are being made to develop vanadium derivatives with high potency and lower toxicity. A synthetic organic compound Bis (maltolato) oxovanadium (IV), with high lipophilicity appears hopeful (Yuen et al., 1993a). Peroxovanadium derivatives are also interesting because of their very high efficacy to mimic the effects of insulin in in vitro (Posner et al., 1994). These compounds have been shown to lower blood glucose levels immediately after injection to diabetic rats (Shisheva et al., 1994) but it is unclear whether they will remain active and non-toxic during chronic oral treatment.
**Effect of Vanadium on Disease Processes**

Vanadium compounds as insulin mimetics in vitro

In 1899, vanadium salts were reported to reduce glycosuria in diabetic patients. Interest in the use of vanadium derivatives for the treatment of diabetes was rekindled when in 1979, vanadium compounds were shown to increase glucose transport and oxidation in adipocytes, to stimulate glycogen synthesis in liver and diaphragm and to inhibit gluconeogenesis in hepatocytes (Tolman et al., 1979). Exogenously added metavanadate mimicked insulin in stimulating hexose uptake (Dubyak and Kleinzeller, 1980), glucose oxidation (Shechter and Karlish, 1980), lipogenesis (Schechter and Ron, 1986) and the inhibition of lipolysis (Degani et al., 1981). These insulin like effects of vanadium were rapidly confirmed and dissociated from its ability to inhibit Na⁺K⁺ ATPase. The half maximally effective concentrations of VO₃⁻ or VO₄³⁻ for mediating the various insulin-like effects range between 0.05 and 0.2 mM. These effects do not seem to be secondary to the inhibition of Na⁺K⁺ ATPase activity. This is because VO₃⁻ over a concentration range of 0.5-1 mM does not inhibit Na⁺K⁺ ATPase activity at all in intact adipocytes (Dubyak and Kleinzeller, 1980; Shechter and Karlish, 1980).

Since then, vanadium salts have been found to mimic most biological effects of insulin in various cell types. In isolated adipocytes, vanadium compounds stimulate glucose uptake and its oxidation (Tolman et al., 1979). They also activate lipogenesis, inhibit lipolysis and increase lipoprotein lipase activity from rat adipose tissue (Ueki et al., 1989). They also activate glycogen synthase in adipose tissue. They exert Ca²⁺ influx in adipose tissue (Clausen et al., 1981) and inhibit Ca²⁺Mg²⁺ ATPase in rat adipocytic plasma membranes (Delfert and McDonald, 1985). Recently it was observed that vanadate was more potent than insulin in stimulating glucose oxidation (¹⁴CO₂ production) from (1-¹⁴C) glucose in rat adipocytes.
Glucose oxidation from (6-14C) glucose was identically stimulated by either insulin or vanadate. \( ^{14}\text{CO}_2 \) production from (1-14C) glucose is a measurement of pentose phosphate shunt activity, whereas \( ^{14}\text{CO}_2 \) production from (6-14C) glucose reflects glycolytic flux. Therefore, vanadate may have a larger effect than insulin on the cellular pentose phosphate shunt activity (Duckworth et al., 1988).

Vanadate inhibited glucose output by 60% from perfused rat liver (Bruck et al., 1991) and increased glycolysis by affecting a variety of enzymes involved in the glycolytic pathway towards increased glucose breakdown (Rodriguez-Gil et al., 1991). Vanadate inhibited glucose-6-phosphatase, stimulated 2,3-bisphosphoglycerate phosphatase and counteracted glucagon effects on 6-phosphofructo-2-kinase activity in rat hepatocytes and adipocytes. Vanadate accelerated glycolysis by inducing L-type pyruvate kinase gene (Miralpeix et al., 1991). It also lowered mRNA levels of phosphoenolpyruvate carboxykinase in hepatoma cells (Bosch et al., 1990). It also activated glycogen synthase and inactivated phosphorylase in hepatocytes (Jackson et al., 1988). It was found to suppress the secretion of apolipoprotein B from rat hepatocytes. It was generally found to promote glycogen deposition in hepatocytes.

In muscles, in vitro effects of vanadium were not comparable to insulin's metabolic actions and might be tissue dependent. Vanadate enhanced glucose uptake, glycogen synthesis and glycolysis to a lesser extent compared to insulin but caused a greater stimulation of lactate and glucose oxidation (Clark et al., 1985). However, unlike insulin, had no effect on protein synthesis or degradation (Clark et al., 1985). Amino acid transport was reported to be enhancing in rat skeletal muscle (Henriksen, 1991; Munoz et al., 1992). Improved glucose transport in peripheral tissue, a major effect of vanadate demonstrated in rat adipocytes (Dubyak and Kleinzeller, 1980), mouse brain (Amir et al., 1987), rat skeletal
muscle (Okumura and Shimazu, 1992) could be attributed to an enhanced translocation of insulin-regulatable transporter (GLUT 4) to the plasma membrane. Vanadate increased glucose transporter expression \textit{in vitro} in NIH 3T3 mouse fibroblasts (Mountjoy and Flier, 1990) and \textit{in vivo} in rat skeletal muscle (Strout et al., 1990).

Unlike the effects of insulin in certain cells, the mitogenic related delayed events of insulin (or EGF) are augmented in the presence of vanadate but are not stimulated alone in the sense that insulin must be constantly bound to its cellular receptors for many hours (Kadota et al., 1986). Hence, intracellular signal(s) for DNA synthesis are transitory and disappear on removal of insulin. Vanadate added to the cells stabilizes the messenger(s) in such a way that the signal does not decrease (Reid and Reid, 1987).

Further studies have indicated that insulin and vanadate share several common features. Vanadate at sufficiently high concentrations maximally stimulates hexose uptake, glucose oxidation and lipogenesis. Furthermore, no increment in stimulation could be achieved by the addition of insulin to vanadate-stimulated cells, and vice versa. In addition, both agents show the same concentration dependency on extracellular glucose. Agents and other conditions which suppress the effects of insulin, such as anticalmodulin drugs, polymyxin B, bicarbonate-depleted buffers and exogenously-added ATP are equipotent in suppressing vanadate-mediated effects. Also, similar rates in the termination of lipogenesis were observed after removing either insulin or vanadate from stimulated adipocytes (Schechter et al., 1988).

\textit{Vanadium Compounds As Insulin Mimics In Vivo}

The properties of vanadium described above prompted \textit{in vivo} investigations of these compounds in animal models of type 1 and type 2 diabetes. Orally administered vanadium has been shown to lower blood glucose in both chemically induced and spontaneously
diabetic rats and mice (Heyliger et al., 1985; Ramanadham et al., 1989a; Heffetz et al., 1990; Ramanadham et al., 1990; Shechter, 1990; Shechter et al., 1990).

Animal models resembling type 1 diabetes mellitus

The first report of insulin mimetic and anti-diabetic potential of vanadium *in vivo* was published by Heyliger et al (1985) wherein normalization of hyperglycemia and improvement of depressed cardiac function without increase in plasma insulin levels of streptozotocin (STZ)-diabetic rats with chronic sodium orthovanadate treatment was reported. This observation demonstrated the ability of vanadium to improve insulin sensitivity. Hyperinsulinaemic clamp studies later confirmed a decreased insulin resistance following vanadium treatment. Since vanadyl sulfate was reported to be 6-10 times less toxic than vanadate, this form of vanadium was extensively investigated for its insulin like effects. Moderate to good diabetic control had been obtained with several vanadium compounds in the STZ-diabetic rats at oral doses between 0.1 and 0.7 mM/kg/day (Meyerovitch et al., 1987; Pederson et al., 1989; Blondel et al., 1990; Sekar et al., 1990; McNeill et al., 1992; Thompson et al., 1993). The dose of vanadium required to achieve good diabetic control varies with the initial diabetic state of the animal (Thompson et al., 1993), the particular vanadium compound given (Cam et al., 1993b; Yuen et al., 1993a), addition of other trace elements (Srivastava et al., 1993) and as yet undetermined individual factors (Bendayan and Gingras, 1989; Cam et al., 1993b). More severely diabetic rats required much higher doses of vanadyl sulphate. Some diabetic rats which were not rendered normoglycemic by low dose treatment with vanadium (<0.5mM/kg/day) nonetheless had significantly lower plasma triglyceride and cholesterol levels and improved glucose tolerance compared to untreated diabetic animals (Yuen et al., 1993a).
Oral administration of vanadate or vanadyl causes a fall in blood glucose levels within 2-5 days. The efficacy of the treatment appears to be inversely related to the severity of diabetes and persists until one year. The treatment also reversed some or all of the following symptoms in treated diabetic animals: hyperphagia, polydipsia (Pugazhenthi and Khandelwal, 1990; Venkatesan et al., 1991), hyperlipidaemia (Mongold et al., 1990) and hypothyroidism (Ramanadham et al., 1989a). Plasma insulin levels were either not affected or were lower than controls in treated diabetic and non-diabetic rats (Cam et al., 1993b; Yuen et al., 1993a). The improvement in glucose homeostasis could not be ascribed to an accelerated glucose excretion as glucosuria was also decreased or to a reduction in intestinal glucose absorption (Madsen et al., 1995) as effects of vanadium were detectable during intravenous and oral glucose tolerance tests as well as in the fasting state.

Chronic treatment of diabetic rats with vanadium restored the ability of insulin to inhibit hepatic glucose production \textit{in vivo} (Blondel et al., 1990) by correcting the abnormal expression of genes coding for the key enzymes of glucose metabolism, thus shifting the gluconeogenic flux to glycolytic flux (Valera et al., 1990; Brichard et al., 1993). Impaired glycogen synthase activity and glycogen reserve were returned to normal (Gil et al., 1988; Bollen et al., 1990). Vanadate also restored the impaired glucose disposal by muscles through correction both of decreased expression of glucose transporter and decreased activity of glycogen synthase (Rossetti and Launghlin, 1989; Blondel et al., 1990; Venkatesan et al., 1991). Vanadate also decreased the high rate of lipolysis (Cam et al., 1993) and promoted lipogenesis, however, the blunted expression and activity of lipogenic enzymes were not modified (Brichard et al., 1994).

Oral vanadyl sulphate, 0.5 g/l reduced sorbitol accumulation (Saxena et al., 1992) and at 1 g/l effectively prevented cataract development in STZ-diabetic rats (Thompson and McNeill, 1993).
Vanadyl sulphate treatment, up to 1.25 g/l in the drinking water, resulted in near normal organ/body weight ratios of lung, heart, liver, kidney and adrenal glands as opposed to the significantly elevated ratios determined in untreated diabetic rats (Dai et al., 1994). Histopathological tests showed no acceleration in morphological abnormalities in a variety of tissues, while overall mortality was 19% in the treated diabetic rats compared to 60% in the untreated diabetic animals. Tissue vanadium levels varied from 6.5-15.1 µg/g in bone, from 3.6-7.3 µg/g in kidney and from 0.2-0.5 µg/g in plasma (Dai et al., 1994).

Cam et al. (1993b) tested the putative prophylactic action of vanadium against the cytotoxic destruction of pancreatic β cells by STZ in STZ-diabetic rats. Irrespective of the delay separating diabetes induction and beginning of the treatment, parameters such as glucose tolerance, adipose tissue function were normalized indicating that insulin mimetic action of vanadium is not due to its protective effect on pancreatic β cell against deleterious effects of STZ.

Concentration dependent effects and the in vivo interaction of vanadyl with insulin was studied by Battell et al (1992) and Ramanadham et al. (1990). Vanadyl sulfate reduced the dose of insulin required by diabetic BB rats, a spontaneous model of type 1 diabetes that does not produce insulin, in a dose dependent manner, but could not totally replace insulin, thus demonstrating insulin-sparing effect of vanadium compounds in vivo (Battell et al., 1992).

Animal models of type 2 diabetes mellitus

Anti-diabetic effects of vanadium have also been investigated in chemically induced and genetic models of type 2 diabetes. Vanadium treatment corrected basal and stimulated hepatic glucose production and peripheral glucose utilization in neonatal-STZ diabetic rats. Vanadium treatment attenuated hyperglycemia,
improved glucose tolerance and hepatic glycogen content in ob/ob (Brichard et al., 1989) and db/db (Pugazhenthi et al., 1991) mice in which a marked insulin resistance leads to overt diabetes despite very high plasma insulin levels (Brichard et al., 1990; Meyerovitch et al., 1991). It also prevented pancreatic exhaustion of insulin. In genetically obese and mildly glucose intolerant fa/fa rats, oral vanadate treatment attenuated hyperinsulinaemia and impaired glucose tolerance (Brichard et al., 1989). Vanadium decreased food and fluid intake, reduced weight gain, attenuated hyperglycemia, hyperinsulinaemia and hyperlipidaemia in Zucker diabetic fatty rats. It restored glucose tolerance and decreased pancreatic insulin depletion. This improvement resulted from a correction of poor sensitivity to insulin of peripheral tissue, particularly muscles (Brichard et al., 1992). Neither the number or affinity of insulin receptor, nor the tyrosine phosphorylation of the receptor itself or of its substrate was affected by vanadate treatment (Lyonnet et al., 1899). It is, therefore, likely that vanadium acts at site(s) distal to the receptor in these models of insulin resistance. It was found to produce the beneficial effects on glucose homeostasis through functional improvement of GLUT 4 transporter (Brichard et al., 1992).

Vanadium Coordination Complexes In-Vivo

Coordination complexes of vanadium (V) and vanadyl (IV) which are recently reported to be insulin mimetics are summarized below.

\[
\text{Bis (maltolato) oxovanadium (IV) (BMOV)}
\]
Review of Literature

Bis (cysteine methyl ester) oxovanadium (IV)

Bis (cysteine, amide N-octyl) oxovanadium (IV) (Naglivan)

Ligandoxobis(peroxo)vanadate (V)  Ligandoperoxovanadium (V)

Figure 6: Coordination complexes of vanadium (V) and vanadyl (IV) recently reported to be insulin mimetics

Ligands have been chosen to impart specific features to the resulting V complexes: improved lipophilicity - vanadyl cysteine methyl ester (Naglivan) (Sakurai et al., 1990), improved oral absorption by passive diffusion – Bis(maltolato)oxovanadium (BMOV) (McNeill et al., 1992), potentiation of in vitro insulin-mimetic effects – peroxovanadate (Kadota et al., 1987; Shisheva and Shechter, 1993b; Posner et al., 1994), or facilitation of transmembranal ion uptake – RL-252 and RL-262 (Shechter et al., 1992).

Naglivan, bis(N-octylcysteineamide) oxovanadium (IV), is insoluble in water. When administered in a suspension of 3% acacia gum by oral
gavage at doses of 0.1-0.3 mM/kg/day, Naglivan was effective in lowering blood glucose of STZ-diabetic rats to near-normal levels but had a very slow onset of action compared to treatment with inorganic vanadium. The advantage over vanadate or vanadyl treatment included lack of weight loss or reduction in food and fluid intake in control animals administered Naglivan (Cam et al., 1993a).

A series of bis(ligand) oxovanadium (IV) complexes were studied in STZ-diabetic rats (Sakurai et al., 1990). The ligands were salicylates, oxalate, malonate, tartrate and cysteine methyl ester. The bis(cysteine methyl ester) vanadyl complex, at a dose of 10 mg/kg body weight (0.2mM/kg), was slightly more effective than the others in lowering blood glucose in diabetic rats when administered intraperitoneally at doses up to 0.2 mM/kg/day, approximately the same as for intraperitoneal treatment with vanadyl sulphate (Sakurai et al., 1990). Oral toxicity tests demonstrated no toxicity at 10 mg/kg/day vanadium (0.2 mM/kg/day); however, at 100 mg/kg/day vanadium (2 mM/kg/day), all test animals died of diarrhoea within 4 days (Sakurai et al., 1990).

BMOV [Bis(maltolato)oxovanadium] (IV), was prepared as a vanadyl complex which would be water soluble, charge neutral and readily available for gastrointestinal absorption by passive diffusion from drinking water solutions (McNeill et al., 1992). BMOV, at a dose of 0.4 mM/kg/day, was effective in reducing blood glucose and lipid levels to near-normal with no diarrhoea and no mortality during the six month study (Dai et al., 1993; Yuen et al., 1993a). The increased absorption of vanadium from BMOV administration was reflected in higher tissue concentrations of vanadium for a similar course of treatment with vanadyl sulfate (Yuen et al., 1993b). An advantage of the apparent increased absorption is the more rapid onset of action of BMOV compared to other vanadium compounds tested so far.

A series of dihydroxamnic acid chelators has been designed as hydrophobic carriers for the vanadyl ion (Shechter et al., 1992). In
assays of lipogenic stimulation in rat fat cells, RL-252 and RL-262 were maximally effective at molar ratios of 10:1 vanadyl sulfate/chelator suggesting a shuttle effect. Compounds were electrically neutral, lipid soluble and optionally chiral and release bound metal ion when treated with aqueous glutathione solutions.

Potentiation of vanadate complexes by addition of hydrogen peroxide was a serendipitous discovery (Kadota et al., 1986; Kadota et al., 1987). New complexes, termed pervanadates, were found to stimulate lipogenesis, inhibit lipolysis and promotes protein synthesis in rat adipocytes at micromolar concentrations (Fantus et al., 1989). Addition of catalase to vanadate solution prior to addition of peroxide abolished the synergism of vanadate with hydrogen peroxide; however, addition of catalase 15 mins after the mixing of vanadate and peroxide preserved the synergism (Kodata et al., 1987), suggesting that complexation of the peroxide to the vanadium has taken place (Posner et al., 1990). Subsequently, a variety of new monoperoxovanadate and diperoxovanadate compounds have been synthesized as insulin mimetics (Posner et al., 1994), including monoperoxopicolinatovanadate and monoperoxo(pyridyne 2,6-dicarboxylato)vanadate which activated insulin receptor kinase activity and inhibited protein phosphotyrosine phosphatase (PPTPase) in rat liver endosomes at 5-80 mM concentrations and also lowered plasma glucose at doses in the mM/kg body weight range. The stimulation of insulin receptor kinase by pervanadate complexes presumably represents the different mechanism of insulin mimesis from vanadate or vanadyl, which appear to have post-IR (or cytoplasmic receptor kinase) stimulatory effects.

Acute studies and glucose clamp investigations

Vanadium's insulin-mimetic effects in vivo have been confirmed by acute and short term studies demonstrating increasing percentage achievement of normoglycemia with increasing doses of vanadium for
variety of compounds (Yuen et al., 1993a). At doses ranging from 16 to 280 μM/kg body weight by intraperitoneal injection, plasma glucose was lowered to less than 8.3 mM within eight hour in progressively more rats (all initial diabetic) as the dose was increased (Yuen et al., 1993a). Tissue levels of vanadium ranged from 0.3-59 μg/gm wet weight for kidney and 0.1-48 μg/ml of plasma.

In short term euglycemic clamp studies indwelling venous and arterial catheters permit maintenance of plasma glucose levels in response to exogenous insulin by infusion of tritiated glucose. Using this technique, sodium metavanadate, 0.2 g/l (Blondel et al., 1989) as well as vanadyl sulfate, 0.5 g/l (Venkatesan et al., 1991) has been shown to suppress hepatic glucose production and improved glucose utilization induced by submaximal or maximal insulin levels, compared to control rats. In partially pancreactomized Sprague-Dawley rats sodium metavanadate, 0.2 g/l (36-39 mM/kg/day) lowered fed and fasted plasma glucose levels without altering plasma insulin levels or affecting body weight gain (Cordera et al., 1990).

Vanadium has also been combined with lithium (Rossetti et al., 1990; Srivastava et al., 1993) and zinc, lithium as well as magnesium in combination (Rossetti et al., 1990) to potentiate the effect of vanadium alone in improving glucose tolerance. Pancreactomized rats given sodium vanadate, 0.05 g/l and lithium carbonate 0.3 g/l in the drinking water for 2-3 weeks prior to euglycemic clamp investigation showed improved glucose uptake and skeletal muscle glycogenic rate, compared to untreated pancreactomized rats (Rossetti et al., 1990). Addition of magnesium sulphate 0.3g/l and zinc sulphate 0.3 g/l to some of the treated rats resulted in further slight but significant improvement in tissue glucose uptake (Rossetti et al., 1990). In a separate experiment, diabetic rats given sodium vanadate 0.05g/l and lithium carbonate 0.3g/l in the drinking water were rendered normoglycemic within four days and had partially restored liver and kidney superoxide
dismutase activities following 16 days of treatment (Srivastava et al., 1993), demonstrating an ameliorative effect on antioxidant status in lithium and vanadate treated diabetic rats.

Human Trials

Recently, limited clinical trials of vanadium compounds have been initiated on human type 1 (IDDM) and type 2 (NIDDM) diabetic subjects. Sodium metavanadate administered for 2 weeks at 125 mg daily in divided doses resulted in significant increases in mean rates of glucose metabolism in 2 out of 5 subjects with type 1 diabetic and 5 out of 5 subjects with type 2 diabetes (Goldfine et al., 1995). In type 1 diabetic patients, vanadium lowered insulin requirements without an effect on C-peptide levels suggesting the absence of an influence on insulin release (Goldfine et al., 1995). Treatment lowered serum cholesterol levels in all subjects. In type 2 diabetic patients, improved insulin sensitivity, enhancement of non-oxidative glucose disposal rates and higher basal MAP and S6 kinases activity in monocytes were recorded. Hepatic glucose production was unchanged. Diarrhoea was the main side effect observed (Goldfine et al., 1995). Treatment with vanadyl sulfate (100 mg/day) for 3 weeks caused improved insulin sensitivity, reduction in hepatic glucose production and increased rate of glucose disposal, all of which were sustained for 2 weeks after treatment was withdrawn (Cohen et al., 1995). This finding is in agreement with some animal studies (Pederson et al., 1989; Becker et al., 1994). In both the studies, incidence of mild gastrointestinal intolerance was observed. Vanadyl therapy for 6 weeks at 100 mg/day also showed similar results i.e. reduced fasting plasma glucose and HbA1C were recorded without an effect on plasma insulin levels. In a more recent study, 25 mg/day vanadyl sulfate, there was no change in glucose and lipid metabolic parameters (Goldfine et al., 1998). Doubling the dose of vanadyl sulfate improved insulin sensitivity without any significant change in plasma glucose levels (Goldfine et al., 1998). There was no increase
in thiobarbituric acid reactive substances at these doses. Though those studies are encouraging, further evaluation of the long term effectiveness of vanadium salts, particularly in type 2 diabetic patients are warranted.

Mechanism of Action

The mechanism of action of vanadium in producing its anti-diabetic effects in vivo is poorly understood and is currently the subject of much investigation. In vitro and in vivo data demonstrate that vanadium affects various aspects of insulin signaling pathway. Vanadium stimulates autophosphorylation of insulin receptor (Ueno et al., 1987; Gherzi et al., 1988) either by activation of tyrosine-kinase (Smith and Sale, 1988) present in the β sub-unit of the receptor itself or by inhibition of phosphotyrosine phosphatase that dephosphorylates the receptor (Swarup et al., 1982; Tracy and Gresser, 1986). These insulin mimetic effects of vanadium could be by virtue of it behaving as a phosphate analog. Other studies however demonstrated that vanadium was equally effective in stimulating glucose metabolism in rat fat cells when half the insulin receptors had been inactivated by insulin over-stimulation (Green, 1993). Furthermore, glucose-lowering effects with oral vanadium treatment were observed while no insulin receptor kinase activity change could be recorded (Mooney et al., 1989) indicating potential post-receptor effects of vanadium, further downstream in the insulin-signaling cascade (Shisheva and Shechter, 1993a; Shechter et al., 1982). Vanadium activated a cytosolic (non-receptor) insulin insensitive protein tyrosine-kinase (Cyt PTK) distinct from IR tyrosine-kinase. This activation was linked to glucose oxidation and lipid synthesis but dissociated from glucose uptake and inhibition of lipolysis. Cyt PTK would be highly selective for vanadium since neither insulin nor isoproterenol, dibutyryl cAMP, okadaic acid, hydrogen peroxide or phorbol ester TPA did affect Cyt PTK activity.
In addition to vanadium, other PTPase inhibitors have also been shown to activate Cyt PTK in adipocytes. It should be noted that insulin mimetic effects of vanadium on hexose uptake and inhibition of lipolysis are not blocked by staurosporine [a blocker of Cyt PTK] indicating that this pathway is not the only means by which vanadium influences cellular physiology (Sekar et al., 1996).

**Figure 7: Putative sites of vanadium action in the insulin signal transduction pathway**

Insulin signal transduction is mediated intracellularly through a complex network of cascades of reversible protein phosphorylations and dephosphorylations. The phosphorylation of the insulin receptor by vanadate is weak, even when the element exerts an antilipolytic action similar to that of insulin. Vanadium also stimulates MAP and S6 kinases that have been demonstrated to be defective in both the basal insulin stimulated state in STZ-diabetic rats (Hei et al., 1994,
Thus insulin resistance associated with long term diabetes may be linked with altered signaling through these kinases and vanadium could rectify the observed defects. Taken together, these finding strongly suggest that vanadium salts bypass the early events of the phosphorylation-dephosphorylation cascade triggered by insulin. This raises the possibility that the element might be able to influence glucose metabolism even when the insulin transduction pathway is not functioning correctly.

When long term effects of vanadium treatment were studied, a persistent hypoglycemic state following vanadium treatment with only minor improvements in pancreatic secretory function was observed. The vanadium-treated rats could sustain an increased sensitivity to circulating insulin even after the treatment was stopped. A possibility could be that vanadium is released from potential tissue storage sites producing the anti-hyperglycemic effects although this seems highly unlikely. Alternatively, Cam et al (1997) suggested that vanadium-induced amelioration of the diabetic state may be partially due to preservation of a functional portion of pancreatic beta cells in the STZ animals. This study showed that a modest increase in β-cell content was crucial to the long-term effect of vanadium even though the total insulin content was still much less than normal. The absence of normal plasma insulin levels strongly suggests the presence of additional actions of vanadium, perhaps at the levels of insulin sensitive tissues (Cam et al., 1997).

**Hypertension**

Oxovanadium compounds cause vasoconstriction and increased arterial blood pressure. Oral treatment of rats with vanadate also increased rat blood pressure (Steffan et al., 1981). However, whether this is due to direct effects of vanadate or not on smooth muscle remains to be determined. Vanadate infusion in dog or cat caused arterial hypertension, increased peripheral resistance and caused a
marked reduction of coronary and renal blood flow. Larger arteries (femoral and carotid) were not constricted (Borchard et al., 1981, Larsen and Thomsen, 1980a).

Carcinogenesis

In mice there has been shown to be a 50% reduction in the ability to induce mammary carcinomas with N-methyl nitroso urea for animals fed vanadyl sulfate (Thompson et al., 1984). The interactions of vanadium with phosphorylation/dephosphorylation processes, especially involving known oncogene products will be related to its effects on carcinogenesis.

Depressive Illness

The levels of vanadium in depressive illness have been examined (Naylor et al., 1987). Vanadium concentrations in hair, whole blood and serum were greater in depressed patients compared to vanadate levels after recovery in the same patients. Renal clearance was lower and mean serum vanadium concentration higher using two different populations of depressed patients compared to controls. Patients taking lithium for depressive illness were found to have lower serum vanadium and cobalt, with elevated serum aluminum (Campbell et al., 1988).