STUDIES ON THE EFFECT OF BORDETELLA PERTUSSIS PREPARATION ON HYPERGLYCAEMIA AND ASSOCIATED RESPONSES INDUCED BY CATECHOLAMINES

THESIS SUBMITTED
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

SUBMITTED BY
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UNDER THE GUIDANCE OF
DR. P. RAMAKRISHNAN

HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
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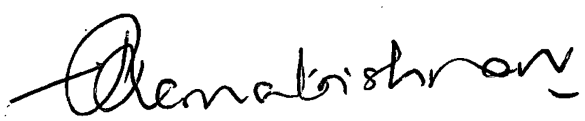

DR. P. RAMAKRISHNAN

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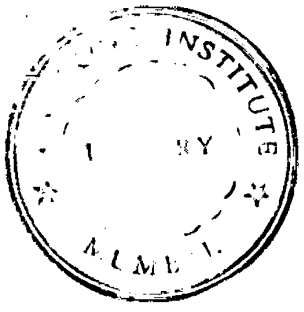
Dedicated to

my father-in-law



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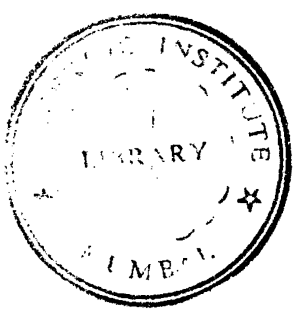

Introduction

Epinephrine and nor epinephrine (also known as adrenaline and noradrenaline) are closely related hormones made and secreted by the medulla of adrenal glands. These are water-soluble amines derived from tyrosine via dopamine which functions as a neuro-transmitter. Epinephrine, norepinephrine and dopamine are called catecholamines (fig. 1), since they may be regarded as derivatives of catechol or 1, 2-dihydroxy benzene.

Among the catecholamines, epinephrine exerts important effects on metabolism. Normally the hormone level in the blood is about 10^{-10} M, but stimuli that alarm the animal galvanize it into readiness to fight or flee causes the concentration of epinephrine to increase almost a thousand fold in seconds or minutes. It prepares the animal for emergency in several ways. It increases the heart output and the blood pressure preparing the cardio vascular system for emergency action. It stimulates the breakdown of liver glycogen into blood glucose, the fuel for anaerobic muscular work and it promotes the anaerobic breakdown of the glycogen of skeletal muscle into lactate via glycolysis, thus stimulating glycolytic ATP formation[1].

Mechanism of action :-

The hormone epinephrine released by adrenal medulla co-ordinates with the cells of different organs to get a characteristic change. It achieves the access to the interior of the cell through specific molecules called the hormone receptors. These receptors are located

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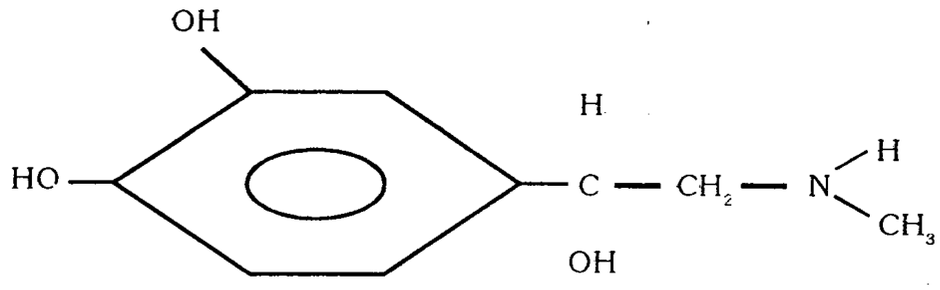
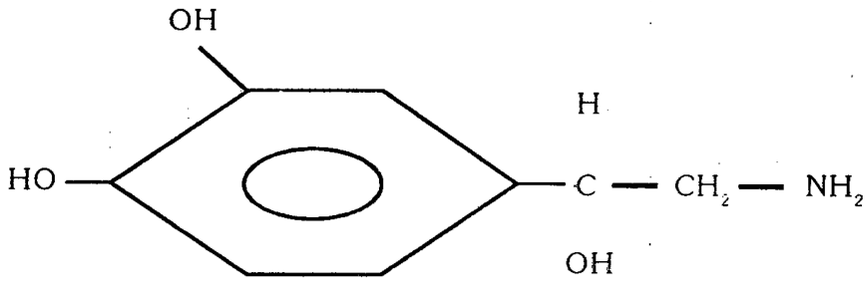
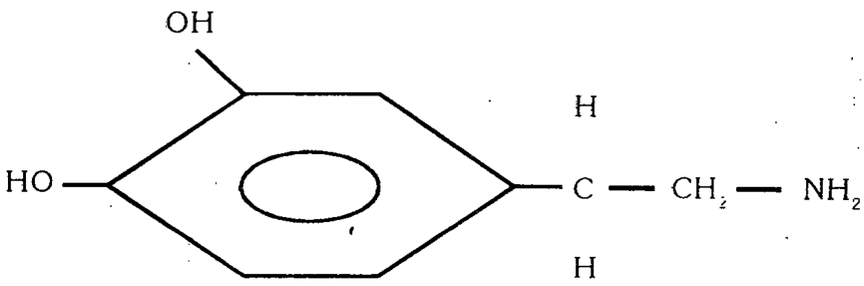


Epinephrine



Nor-epinephrine



Dopamine

Fig. 1 : Catecholamines

on the outer surface of the target cells. These receptors have high specificity and affinity for the given hormone molecule. Once the hormone receptor on the target cell is occupied by the hormone molecule, the receptor undergoes a characteristic change which forms or sets free an intracellular messenger molecule, often called the second messenger. This messenger conveys the signal from the hormone receptor to some enzyme or molecular system in the cell that carries out the instruction brought in by the hormone. The intracellular messenger either regulates a specific enzymatic reaction or causes an inactive gene to be expressed.

Dopamine binds to the dopaminergic receptors while epinephrine and nor-epinephrine binds to the adrenergic receptors.

Dopaminergic receptors :

Dopaminergic receptors exist in certain peripheral vascular beds and visceral smooth muscle as well as the peripheral and central nervous system (CNS) and are clearly distinct from adrenergic receptors[2].

Adrenergic receptors :

Adrenergic receptors are sites on cell-membrane that specifically recognizes and binds epinephrine, norepinephrine and a variety of synthetic catecholamine agonists and antagonists that modulate the physiological and pharmacological actions of those substances[2]. These receptors are transmembrane proteins with extracellular amino

terminus and intracellular carboxy terminus. Each of their 7 hydrophobic region span the cell-membrane. Although they exhibit significant amino-acid homology, differences in 5^{th} and 6^{th} segment determines specificity of agonist binding. Difference in 5^{th} and 7^{th} segment determines whether receptor is coupled to Gs and Gi (guanyl nucleotide binding) protein. Stimulation of Gs protein increases adenylate cyclase and stimulation of Gi protein decreases adenylate cyclase[3].

The studies of sympathetic activation suggested that there were two classes of responses, excitatory and inhibitory. In 1948 Ahlquist postulated two distinct adrenergic receptors, which he designated as α (alpha) and β (beta)[4]. Physiological responses have thus been characterized as alpha or beta on the basis of different agonist potency and antagonism by specific α or β receptor blocking agents[5]. Distinct sub-types of both α and β adrenergic receptor exist[6].

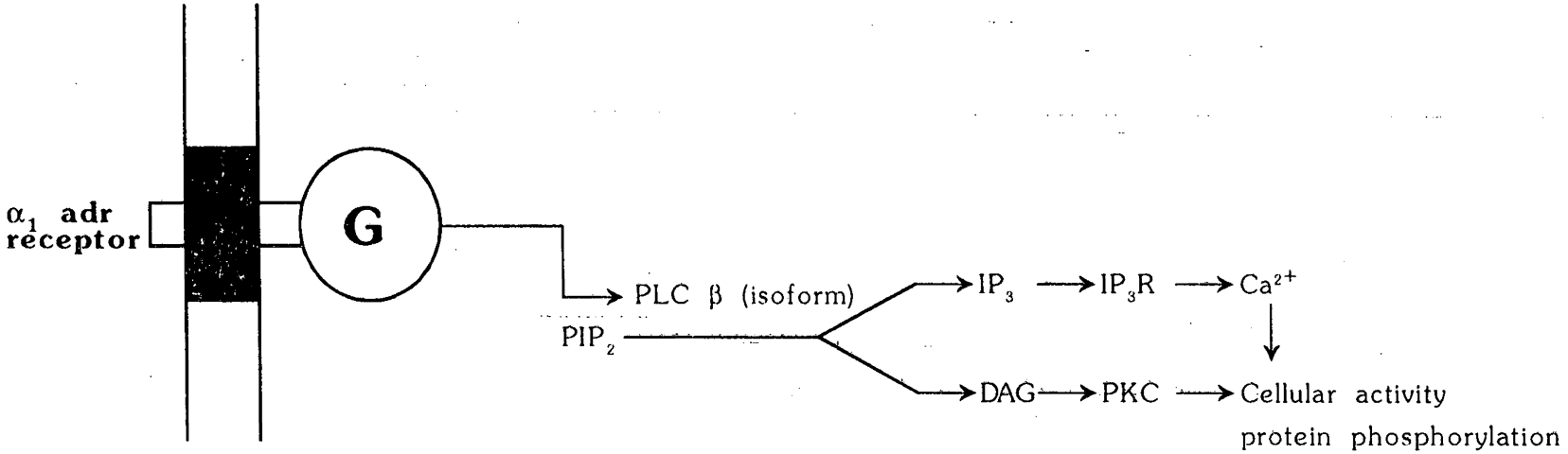
Alpha adrenergic receptor :

Alpha adrenergic receptor mediates a variety of responses including vaso constriction. Epinephrine and norepinephrine are potent non-selective agonists of the α receptor. There are two types of α receptors α_1 and α_2.

The α_1 sub-type mediates a wide variety of alpha effects. It acts through mobilization of intracellular calcium from endoplasmic stores

and in some tissues an influx of calcium (Ca^{2+}) from extra cellular fluid (ECF). This increase in intracellular calcium is thought to result from activation of phospholipase C which results in breakdown of phosphatidyl inositol as given in fig. 2. The diacylglycerol (DAG) thus formed activates protein Kinase C and Inositol triphosphate (IP_3) causes release of Ca^{2+} from intracellular stores thus activating a group of Ca^{2+} and calmodulin-sensitive protein kinases. It causes stimulation of the hepatic enzyme *phosphorylase a* and inhibition of the enzyme glycogen synthase. Phenylephrine, methoxamine are selective agonists of α_1 receptor[2] and prazosin is its selective antagonist[7]. The α_1 receptors are located on vascular smooth muscle, where it mediates contraction. It also causes relaxation of intestinal smooth muscle and increases the contractile force of the heart. In liver it causes glycogenolysis.

The α_2 receptor acts via adenylate cyclase-cyclic AMP system. It inhibits the enzyme adenylate cyclase and thus prevents the cyclic AMP accumulation which results in a number of metabolic and physiological changes. The α_2 receptor causes activation of receptor operated potassium (K^+) channels[7], inhibition of voltage sensitive Ca^{2+} channels[7], along with its ability to inhibit adenylate cyclase. Clonidine and α methyl norepinephrine are its selective agonist and yohimbine is its selective antagonist. The α_2 receptors are located on cholinergic neurons within the gut where it prevents the release of acetyl choline[2]; on CNS neuron where it regulates cardiac function[2]; on



Key

PIP_2 = Phosphatidyl inositol

PLC β (isoform) = PhospholipaseC-β

IP_3 = Inositoltrisphosphate

DAG = Diacylglycerol

IP_3R = IP_3 Receptor

PKC = Protein Kinase C

Fig. 2 :- The α_1 adrenergic receptor mediated pathway.

adrenergic neurons where it prevents the release of norepinephrine from those neurons; on pancreatic β cells where it decreases the insulin secretion; and on vascular smooth muscle, where it mediates contraction.

Beta adrenergic receptors :-

The beta (β) receptor mediates cardiac stimulation, broncho-dilation and vaso-dilation. Sub-types of β receptor are designated as β_1 and β_2.

The β receptor agonist stimulates the enzyme adenylate cyclase, leading to an increase in cyclic AMP which serves as the second messenger[2]. It activates protein kinase which catalyse the phosphorylation of a variety of proteins which cause a characteristic metabolic and physiological change within the cell.

Epinephrine and isoproterenol are the non-selective β agonists. Nor-epinephrine is a potent agonist of the β_1 receptor (equivalent to epinephrine) but a weak agonist of the β_2 receptor. Non-selective β antagonist include propranolol, alprenolol, nadolol and timolol. The β_1 receptor mediates cardiac stimulation and lipolysis, where as the β_2 receptor mediates broncho dilation, vasodilation, prejunctional stimulation of nor-epinephrine release from sympathetic neurons, glycogenolysis and uptake of potassium (K^+) ions in skeletal muscle, glycogenolysis and gluconeogenesis in the liver and secretion of insulin from the β cells of pancreas.

The responsiveness of peripheral effector tissues to adrenaline stimulation may be modified substantially by alteration in temperature, chemical composition of the plasma and circulating levels of different hormones. These changes in responsiveness may originate at (i) the adrenergic nerve terminal where variety of factors influence amount of neurotransmitter release; (ii) the adrenergic receptor level, where alteration in receptor number may occur, (iii) post receptor sites so that relationship between receptor occupancy and physiological responses is modified. Alteration in adrenergic (adr) receptor number have been noted in a variety of physiological and pathophysiological states[8, 9, 10]. In some of these states changes in receptor number appears to account for the altered sensitivity to catecholamines. The term homologous regulation refers to alterations in adr. receptors induced by adrenergic agonist. Heterologous regulation designates the alteration in adr. receptors from environmental changes that do not involve agonists[9, 11].

Homologous regulation :-

Desensitization is a phenomenon ,where by prolonged exposure of an effector tissue to an agonist results in progressive diminution in response. Exposure of both α and β adr. receptors to agonists diminishes adr. receptor number since change in receptor number appears to correlate with loss of physiological response. This agonist induced decrease in receptor number referred to as down regulation contributes

to desensitization[10, 12]. For β receptor down regulation involves partially reversible internalization of receptors[11, 8] in frog erythrocytes[8, 13] and other isolated cell systems[11]. Internalization of β receptor following chronic agonist exposure results in increased cytosolic binding of β receptor ligands, after exposure to agonists, but not to antagonists[13]. The lost receptor also reappear after removal of the agonist, presumably representing the reversal of internalisation process.

Super-sensitivity is also observed in the β agonist which is noted after propranolol withdrawal[14], since maximal physiological response can be demonstrated when only a small portion of β receptors are occupied[11]. Interaction of agonist and receptor follows the law of mass action; increased number of adr. receptors increase the likelihood of agonist receptor interaction thus resulting in a shift in dose response relationship to the left indicating an increase in sensitivity[9] Thus the presence of spare receptor implies that the alteration in receptor number will be translated into alterations in sensitivity to the agonist. Infact alteration in the receptor number correlates with changes in sensitivity to catecholamines in a wide variety of tissues. Thus alteration in receptor number and receptor occupancy are only two among many factors that determine the physiological response.

Heterologous regulation :-

Factors other than adrenergic agonist alter adr. receptor function and sensitivity to catecholamines. At low environmental temperature alpha adrenergic responses are potentiated[15, 16]. A variety of hormones affect adrenergic responsiveness some of which may be mediated by alteration in adr. receptors. Thyroid hormone potentiate β adr. responses and diminish α adr. responses. Estrogen increases and progesterone decreases number of α adr. receptors in rabbit uterus[17, 18]. Estrogen also increases the affinity of α_1 adr. receptor on rat vasculature for catecholamine and decreases number of α_2 adr. receptors on platelets.

Recently different sub-types of α and β receptors have been cloned. The α_1 receptor comprises of α_{1A}, α_{1B}, α_{1D} types of receptors. The α_2 receptor comprises of α_{2A}, α_{2B} α_{2C} types and the β receptor comprises of β_1, β_2 and β_3 types of receptors. The chromosome location and tissue localization has been found out but distinction in their mechanism of action has not been clearly defined yet[7].

Metabolic effects of catecholamines :-

Some of the metabolic effects of catecholamine related to carbohydrate metabolism are discussed here with respect to organs and tissue.

Liver :-

Catecholamines in liver promote hepatic glucose output by activating glycogenolysis and acclerating gluconeogenesis, while simultaneously inhibiting glycogen synthesis. Interaction of catecholamines with the β adrenergic receptor activates the well-known sequence involving stimulation of adenylate cyclase, generation of cyclic AMP and initiation of the cAMP-dependent enzymatic cascade leading to conversion of glycogen phosphorylase from inactive to active form[2]. This effect of epinephrine was among those reported by Rall and Sutherland in their description of adenylate cyclase[19]. While it appears that glycogenolysis in liver is mediated by cAMP, recent evidence indicate that the effect of catecholamines on glycogenolysis in the perfused rat liver were mediated by both a β and an α mechanism [20]. The α mechanism was not associated with changes in cAMP levels and epinephrine could stimulate gluconeogenesis by an α-mechanism[21, 22]. The catecholamine stimulation of gluconeogenesis was blocked by α-blocking agents such as phentolamine and dihydroergotamine, but not by the β-blocker propranolol which suppressed the increase in cAMP. Furthermore the catecholamine stimulation of glucose synthesis was suppressed by α-blockers even though cAMP levels remained elevated. In addition isoproterenol, a pure β agonist, elevated cAMP levels but had no effect on gluconeogenesis. Further ,phenylephrine, a synthetic catecholamine and α-agonist, also increased gluconeogenesis but did not elevate cAMP.

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Thus the α-effect of catec holamine in rat hepatocytes is not associated with an activation of cAMP-dependent protein kinase[23]. It has been suggested by several laboratories that the α- component of catecholamine on glycogenolysis in liver is mediated by alteration in Ca^{2+} influx[24] .

A variety of factors affect the relative contributions of alpha and beta adrenergic receptor mechanisms to the hepatic glucose production including extra-cellular glucose, steroids, glucorticoids, endotoxin and cholestasis[25, 26, 27, 28, 29]. Amino-acid uptake into the liver and perhaps the lactate entry also, is augmented by alpha agonists; effects that increase the availability of substrate for gluconeogenesis.

Species differences also exist. Alpha-adrenergic effects predominate in rats (especially males) whereas beta-adrenergic effects predominate in dogs and humans. Alpha-adrenergic stimulation can enhance hepatic glucose production in humans[30].

Catecholamines as noted previously can suppress insulin and stimulate glucagon secretion in the pancreas. Such changes in glucagon and insulin augment the direct effects on catecholamines on hepatic glucose production. Catecholamines generally diminish hepatic blood flow[31]; and additional effect of glucagon may be to lessen catecholamine induced hepatic arterial vaso constriction[32]. Sympathetic innervation are present in the hepatic parenchyma. This is maximum in the human liver and least in rat or mouse liver. The importance of locally

released nor-epinephrine as compared with circulating epinephrine in the regulation of hepatic metabolism is uncertain. Among the circulating catecholamines, epinephrine in particular, doubtlessly plays a major physiological role. Hepatic glucose out put increases during infusions of epinephrine that produces high but physiologically attainable, arterial levels[2, 33].

White Adipose tissue :-

Catecholamines stimulate lipolysis by activating hormone-sensitive (triacyl glycerol) lipase, the enzyme that cleaves triglyceride into fatty-acids and glycerol within adipose tissue[2]. The cellular processes mediating such response involves interaction of catecholamines with the β_1 adr receptor followed by activation of adenylate cyclase and phosphorylation of the inactive lipase by a cAMP-dependent protein kinase[2]. Removal of phosphate from the lipase by a phosphatase inactivates the enzyme. In addition to β mediated lipolysis, catecholamines exert an antilipolytic effect via an α_2 receptor mechanism[2]. The α_2 -adrenergic sensitivity in adipose tissue differs among species, as do the lipolytic responses to catecholamines. Even within the same individual, fat cells from different locations exhibit different proportion of α and β adr receptors and variable rates of lipolysis in the presence of catecholamines[34]. In addition, numerous environmental and hormonal factors affect adipose tissue responsiveness to catecholamines, insulin being the most prominent.

Insulin exerts an overriding antagonistic effect upon catecholamine-mediated lipolysis; thus suppression of insulin secretion is an important component of catecholamine effects on fat mobilization. Nutritional state, thyroid function, obesity and age also influence the adipocyte response to catecholamines[2].

Muscle :-

Catecholamine stimulation of glycogenolysis in muscle occurs via beta-receptor activation utilizing cyclic AMP as second messenger[2]. Unlike the situation in liver or adipose tissue, alpha-receptor mechanisms do not affect this process, atleast in skeletal muscle[35, 36]. Since muscle lacks the enzyme glucose 6-phosphatase, the glucose 6-phosphate produced by glycogenolysis is metabolized to lactate before its release into the circulation[2]. Catecholamines also enhance free fatty acid entry and mobilizes triglyceride contained in muscle. In skeletal muscle, this response is β receptor mediated. The effect of catecholamines on muscle glycogen metabolism are antagonized by insulin. The precise role of catecholamines in regulation of muscle protein metabolism is uncertain. Catecholamines, via a beta-receptor mechanism, diminish protein degradation but increase oxidation of branched chain amino-acids in cardiac and skeletal muscle[37, 38]. Metabolism of these amino-acids elevates ammonia content in muscle, part of which is transferred to alpha-ketoglutarate with formation of glutamate and glutamine[39, 40]. Gut takes up the glutamine and

releases it as alanine thus supplying it as a gluconeogeneic precurrsor to the liver[41].

Endocrine pancreas :-

Although the secretory activities of the endocrine pancreas is governed predominantly by delivery of substrates particularly glucose and aminoacids, catecholamines influence this function. Sympathetic (and para sympathetic) nerve fibres are in close proximity to all islet cell types, and alteration in insulin and glucagon secretions occurs in response to pancreatic nerve stimulation or administration of adrenergic agonists[2]. β_2 adr. receptor stimulation transiently increases secretion of both insulin and glucagon, whereas α_2 adr. receptor stimulation suppresses insulin secretion. However the effect of α-receptor mechanisms on glucagon secretion is uncertain. Although α-receptor-mediated inhibition of insulin secretion usually predominates over β-receptor mediated stimulation, various factors including glucose, potassium, calcium and thyroid hormone and also whether the agonist is epinephrine or nor-epinephrine, affects the balance between α and β-adrenergic responses[42, 43, 44, 45].

Maintenance of the plasma glucose concentration is critical to survivalbecause plasma glucose is the predominant metabolic fuel utilized by the central nervous system under most conditions. The central nervous system cannot synthesize glucose, store it more than a few minutes supply, or concentrate it from the circulation. Hypoglycaemia

can cause profound brain dysfunction. The glucoregulatory systems have therefore been evolved to prevent or correct hypoglycaemia. It includes the hormones glucagon, epinephrine, cortisol and growth hormone. The plasma glucose concentration is normally maintained within a relatively narrow range of 70-130 mg per 100 ml, despite wide variations in glucose influx and efflux such as those that follow meals and occur during exercise.

Glucose Metabolism :-

Glucose is derived from three sources which are (i) intestinal absorption following digestion of dietary carbohydrates; (ii) glycogenolysis, breakdown of glycogen, the polymerized storage form of glucose and (iii) gluconeogenesis, the formation of glucose from precurrsors including lactate, pyruvate, amino acids and to a lesser extent glycerol.

Although most tissues have the enzyme system required to synthesize glycogen (by glycogen synthase) and to hydrolyze glycogen (by *phosphorylase a*), only the liver and kidney contain the enzyme glucose 6-phosphatase necessary for the release of glucose in the circulation. They also contain the enzymes necessary for gluconeogenesis.

There are multiple potential metabolic fates of glucose transported into the cells. These are storage as glycogen, glycolysis to pyruvate which can be reduced to lactate, transaminated to form alanine or converted to acetyl-coA which in turn can be oxidised to CO_2 and

H_2O via the TCA cycle or utilized for ketone-body (aceto-acetate, β-Hydroxy butyrate) synthesis, and finally released into the circulation.

The liver is remarkably flexible[46]. It is for the practical purposes the sole source of endogenous glucose production. Renal gluconeogenesis and glucose release contribute substantially to the system glucose pool only during prolonged starvation. Under conditions of high glucose out put, the energy needs of the liver are largely provided by the β-oxidation of fatty acids. On the other hand the liver can also be an organ of net glucose uptake with storage as glycogen, which either can remain in the liver or be transported to other tissues.

Muscle can store and utilize glucose, the latter primarily through glycolysis to pyruvate, which can be reduced to lactate or transaminated to alanine before the transport to the liver. During fasting, muscle can reduce its glucose uptake virtually to zero, oxidize fatty-acids for its energy needs and through proteolysis, mobilize amino-acids for transport to the liver as gluconeogeneic precursors; adipocytes also decrease glucose utilization (for fatty acid synthesis) and satisfy energy needs from the β-oxidation of fatty acids.

Other tissues such as the formed elements of the blood and the renal medulla do not have the capacity to decrease glucose utilization upon fasting and therefore produce lactate at relatively fixed rates.

Glucose is the predominant metabolic fuel utilized by the brain under most conditions. It undergoes terminal oxidation to CO_2 and H_2O in the brain. When ketones are plentiful in the circulation, they can support the energy needs of the brain and reduce its glucose utilization[47].

The hypo-function of glucose metabolism results in hypoglycaemia, a condition in which the blood sugar levels go below 60 mg per 100ml. Normally it occurs during excessive starvation or when all the glycogen stores are depleted, after excessive exercise. Abnormally it could occur due to some insulin producing tumour or on external administration of insulin or oral hypoglycaemic agents as in diabetic patients.

Hyper-glycaemia occurs when there is diminished utilisation of glucose and release of excess glucose from the liver. Hyper-function of some of the endocrine glands as the anterior-pituitary and adrenal-cortex causes hyperglycaemia. Lack or diminished secretion of insulin is the major factor which produces hyper-glycaemia and glycosuria as in diabetes mellitus.

Under normal conditions a number of hormones like glucagon, epinephrine, and cortisol are secreted by its respective glands to restore the lowered blood sugar. The release of glucose is achieved by glycogenolysis and gluconeogenesis in the liver. Epinephrine is released under physiological stress such as hypoxia, hypotension, cold,

fatigue and shock. It serves as a emergency hormone by rapidly causing lipolysis and glycogenolysis along with gluconeogenesis. Its effect are mediated through the α and β adr. mechanisms in humans, although the α adr. stimulation of hepatic glucose production in humans[30] and in rats[29] has also been reported. It achieves hyperglycaemia by decreasing glucose uptake in muscles and by inhibition of insulin secretion. The two mechanisms glycogenolysis and gluconeogenesis which maintain the levels of extra cellular glucose are controlled by various hormones and are in itself a complex mechanism involving many enzymes and co-factors.

Glycogenolysis :-

It is a process by which liver glycogen is broken down to glucose 1-phosphate by the enzyme *phosphorylase a*. The glucose 1-phosphate is then converted to glucose 6-phosphate by the enzyme phosphoglucomutase which is common to glycogen synthesis and glycogenolysis. The glucose 6-phosphate is converted to free glucose by glucose 6-phosphatase This enzyme is absent in muscle and therefore muscle glycogenolysis does not yield free glucose, but produces lactate which is the metabolite of the glycogenolysed glucose. This lactate is used up to synthesize glucose by the liver.

Glycogen :-

It is the storage form of polysaccharide in the liver and muscle. It may attain upto 10% of the wet weight of liver and 1-2% of muscles in fed conditions. Glycogen in any tissue is not a static quantity. It is constantly being used up or resynthesized. Liver glycogen is first to be formed and is also the first to be mobilized. Muscle glycogen is much slower to move[48].

In both muscle and liver, glycogen has the function of being a reserve of D-glucose units. Liver glycogen can be used to replenish D-glucose in the blood between meals and during excercise. In muscle however, the enzyme glucose 6-phosphatase is lacking and hence the D-glucose 6-phosphate produced by the breakdown of glycogen is metabolized via the glycolytic pathway to provide ATP that acts as the fuel for muscular contraction. The amount of ATP that can be generated from the muscle glycogen is sufficient for a short time. Prolonged exercise requires utilization of other sources of energy e.g triglycerides[49].

Glycogen Phosphorylase :-

Phosphorylase exists in two forms : *phosphorylase b*, a dimer of Mol. wt. 185,000 essentially active in the presence of AMP and *phosphorylase a*, a tetramer of mol. wt 370,000 active in the absence of this nucleotide[49].

The liver phosphorylase is activated by glucagon and epinephrine. The former hormone would act by stimulating the enzyme adenylate cyclase present in the liver cell-membrane[50], thus causing an elevation of cAMP and a subsequent activation of cAMP - dependent protein-kinase, which phosphorylates the enzyme phosphorylase kinase which in turn phosphorylates the enzyme *phosphorylase b*, converting it into the active *phosphorylase a* form. The enzyme phosphorylase kinase itself can be activated in two main ways : by phosphorylation catalysed by a cAMP-dependent protein kinase as described above or by Ca^{2+} mediated stimulation. Ca^{2+} ions in the concentration range of 0.1 to 1.0 μmol dm^3 can activate phosphorylase kinase in the presence of Mg^{2+} and ATP. This activation occurs by a process of autophosphorylation in which the enzyme catalyses its own phosphorylation at the sites that are distinct from those that are phosphorylated by the action of cAMP dependent protein kinase[49].

In contrast to glucagon, epinephrine acts by increasing the influx of Ca^{2+}, thus activates phosphorylase kinase by bypassing the cAMP dependent protein kinase step[51].

Degradation of glycogen is affected through the action of a group of enzymes which include glycogen phosphorylase, debranching enzyme system, phospho glucomutase and glucose 6-phosphatase as shown in fig. 3. Phosphogluco mutase being common to both glycogeno-synthesis

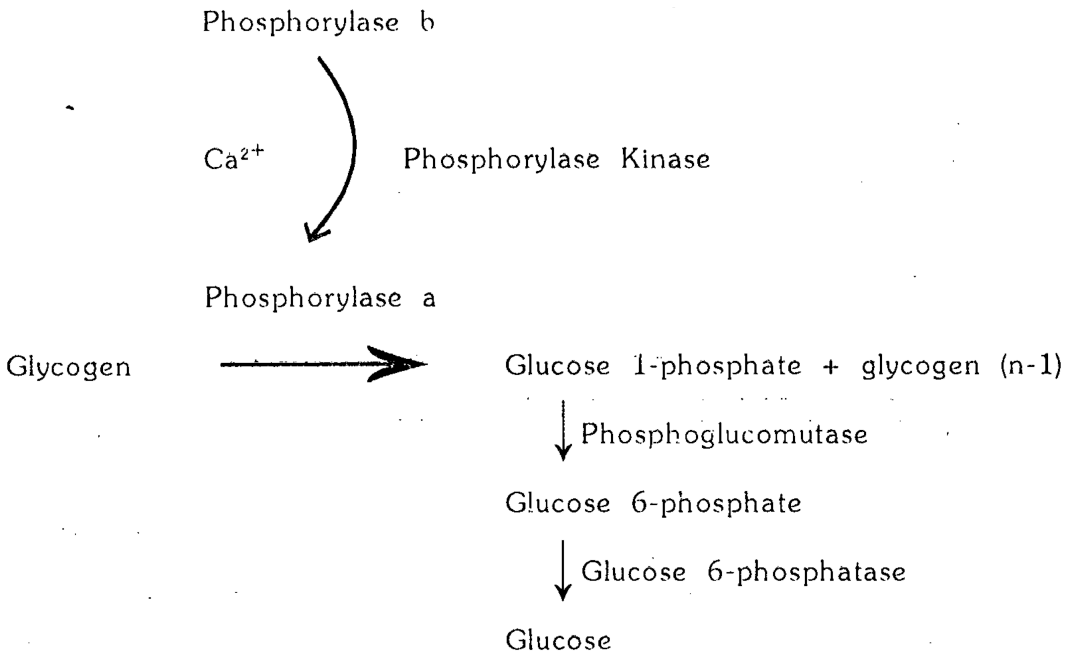


Fig. 3 Glycogenolysis in rat liver

and glycogenolysis. Glucose 6-phosphatase is common to glycogenolysis and gluconeogenesis.

The enzyme *phosphorylase a*, in the presence of orthophosphate affects the phosphorolytic disruption of alpha -1, 4-glucosidic linkages at the non-reducing end of glycogen chains, which are thus progressively shortened, while glucose-1-phosphate is released[51].

Phosphorylase acting *in vivo* favours the reaction in the direction of glycogen degradation[51]. This is confirmed by the observation that hormonal stimulation of phosphorylase always results in an increase in glycogen degradation and by the fact that when phosphorylase activity is deficient because of a genetic enzyme defect, only glycogenolysis is impaired while glycogen synthesis is normal as indicated by the tendency towards glycogen accumulation[51, 52].

Liver phosphorylase is a protein distinct from muscle phosphorylase as shown by the immunological difference between the two enzymes[53]. *Phosphorylase b* is converted to *phosphorylase a* by the enzyme phosphorylase kinase and reverse conversion from the active (*phosphorylase a*) to inactive (*phosphorylase b*) form is accomplished by the enzyme phosphorylase phosphatase[54, 55]. This enzyme is inhibited by presence of fluoride ions *in vitro*[49]. *Phosphorylase a* is inactivated by glucose. It achieves so by stimulating the enzyme phosphorylase phosphatase, thus decreasing glycogenolysis. *Phosphorylase b* is active under conditions when AMP

concentration is high and ATP and D glucose-6-phosphate concentrations are low.

It has been estimated that in the normal resting liver, about 60% of the enzyme is in the active form[56]. *In vivo* experiments have shown that in rat liver the rate of formation of glucose 1-phosphate by phosphorylase is exceedingly lower than the rate of the next reaction in the glycogenolytic sequence i.e the reversible conversion of glucose 1-phosphate to glucose-6-phosphate catalyzed by phosphoglucomutase[51]. These and other data[57] indicate that phosphorylase is rate-limiting enzyme in glycogenolysis.

Phosphorylase a accounts for less than 10% of the total activity in normal human muscle and is increased in muscle with glycogen accumulation[51]. Phosphorylase in muscle exists in both active and inactive forms. Total phosphorylase activity in fast muscle fibre is about 2.5 fold higher than in slow fibres[51]. Phosphorylase also exists in 2 forms in smooth muscle, where the activity is about 10% of that in rabbit skeletal muscle[58]. Phosphorylase in rat kidney shows an activity equal to 35% of that in liver[51].

Regulation by Hormonal Factors :-

Catecholamines :-

Catecholamine activates hepatic glycogen phosphorylase and inactivates glycogen synthase[59]. While this effect might be mediated

by stimulation of adenylate cyclase and consequent cAMP production, several findings suggest a different mechanism, not involving a rise in cAMP[60, 61] It has been observed that while glucagon raises cAMP concentration in rat hepatocytes about 30 -fold, epinephrine (plus theophylline) raises cAMP level only about two fold[62]. Moreover, adenylate cyclase from rat liver plasma-membrane has been reported to be unresponsive to epinephrine[51] or to be influenced in its response to epinephrine by the age, sex and steroid hormones[63] and by calcium[64]. Hepatic adenylate cyclase has also been suggested to be formed by two seperate enzymes, one responsive to epinephrine and one to glucagon[65]. Recent evidence indicates that epinephrine acts through activation of alpha-receptors and activates phosphorylase and inactivates glycogen synthase without causing change in cAMP or activation of cAMP-dependent protein kinase[59]. It is relevant that studies with perfused rat liver suggests that adrenergic receptors in liver cannot be readily classified into an α or a β receptor[61]. Catecholamine activate muscle and heart phosphorylase by increasing the production of cAMP involving β-adrenergic receptor. Ca^{2+} also plays a role in glycogenolysis by activation of the enzyme *phosphorylase b* kinase. The glycogen broken down provides metabolic fuel for muscle contraction[51]. Catecholamines reduce muscle glycogen synthase activity.[66]

Insulin :-

Insulin exerts a profound effect on glycogen metabolism. Administration of insulin to alloxan diabetic rat results in a several-fold increase in hepatic glycogen synthase activity within the first 6 hours of treatment. This increase in enzyme is completely inhibited by puromycin, thus suggesting that the increase in the amount of enzyme is due to its synthesis.[67]

Intravenous infusion of insulin in dogs causes an increase in the I (active) form of glycogen synthase in liver, probably by favouring the conversion of the D (less active) to the I (active) form of the enzyme[51, 68], while at the same time causing inactivation of phosphorylase. These effects are rapidly reversed by glucagon administration[68]. Insulin also promotes glycogen synthase activation in skeletal and cardiac muscle. It has been shown that insulin completely blocks *in vivo* the glucagon stimulated glycogen breakdown by inhibiting the *phosphorylase a* activity[69].

Gluconeogenesis :

Gluconeogenesis is the process whereby lactate, pyruvate, glycerol and certain amino-acids are converted to glucose and glycogen. The liver is the major site of gluconeogenesis, although the kidney becomes important during prolonged starvation. The most important function of gluconeogenesis is the maintenance of blood

glucose levels during times when food intake is restricted and/or glycogen stores are depleted. It is the means whereby the lactate that is produced by glycolysis in erythrocytes and in exercising muscle is reconverted to glucose. Similarly, it conserves the glycerol that is released during lipolysis in the adipose tissue and the alanine produced by amino acid metabolism and glycolysis in the muscle.

Gluconeogenesis also contributes significantly to the utilization of amino acids, which are either absorbed from the alimentary tract or released during protein breakdown in muscle or other tissues. When conditions of metabolic acidosis exist, such as during times of prolonged starvation, the contribution of renal gluconeogenesis to the total glucose produced increases from less than 20% to about 50%[70]. At the low pH, the conversion of glutamate and α-ketoglutarate to glucose is enhanced. This inturn leads to an increase in NH_3 production which is used to counteract the acidosis[71, 72]

Lactate, pyruvate and various gluconeogeneic amino acids are converted to glucose in the hepatocyte. Amino acids and probably lactate enter the cell by various carrier-mediated plasma membrane transport systems. Lactate and most amino-acids are converted to pyruvate in the cytoplasm of the cell, pyruvate enters the mitochondria by a transport system and is converted to oxaloacetate by the enzyme pyruvate carboxylase or to acetyl-Co A by pyruvate dehydrogenase. The fate of the mitochondrial oxalo acetate varies in

different species, depending largely on the distribution of the enzyme phosphoenol pyruvate carboxy kinase (PEP-CK) between cytoplasm and mitochondria.[73] In the rat and mouse liver, it is converted to malate and/or aspartate, and these metabolites are transported to the cytoplasm, where they are reconverted to oxaloacetate. The oxaloacetate is then converted to phosphoenol pyruvate by the enzyme PEP-CK, which is a cytosolic enzyme in rat and mouse liver. This complicated series of interconversions is apparently necessary because the mitochondrial membrane is presumably impermeable to oxaloacetate.[74] In pigeon, chicken and rabbit liver, PEP-CK is in the mitochondria and oxaloacetate is directly converted to phosphoenol pyruvate and then transported to the cytoplasm. In the human, guinea-pig, sheep and cow liver, the enzyme is present both in the mitochondria and the cytoplasm, but the fraction of total phosphoenol pyruvate(PEP) synthesis at each side is not as yet answered. Cytosolic PEP is then converted to glyceraldehyde 3-phosphate by reversal of glycolysis. (fig. 4). 2 moles of the triose phosphate formed are combined by the enzyme aldolase to yield fructose 1,6-biphosphate. This is dephosphorylated to yield fructose 6-phosphate and is converted by glucose phosphate isomerase to glucose 6-phosphate which is dephosphorylated to yield free glucose. Since 2 moles of triose phosphate are needed to form 1 mole of hexose 6-phosphate, the enzymes in the triose section of gluconeogeneic pathway should be twice than that in the hexose section of gluconeogenesis. In the triose section NADH is required at the level of enzyme glyceraldehyde

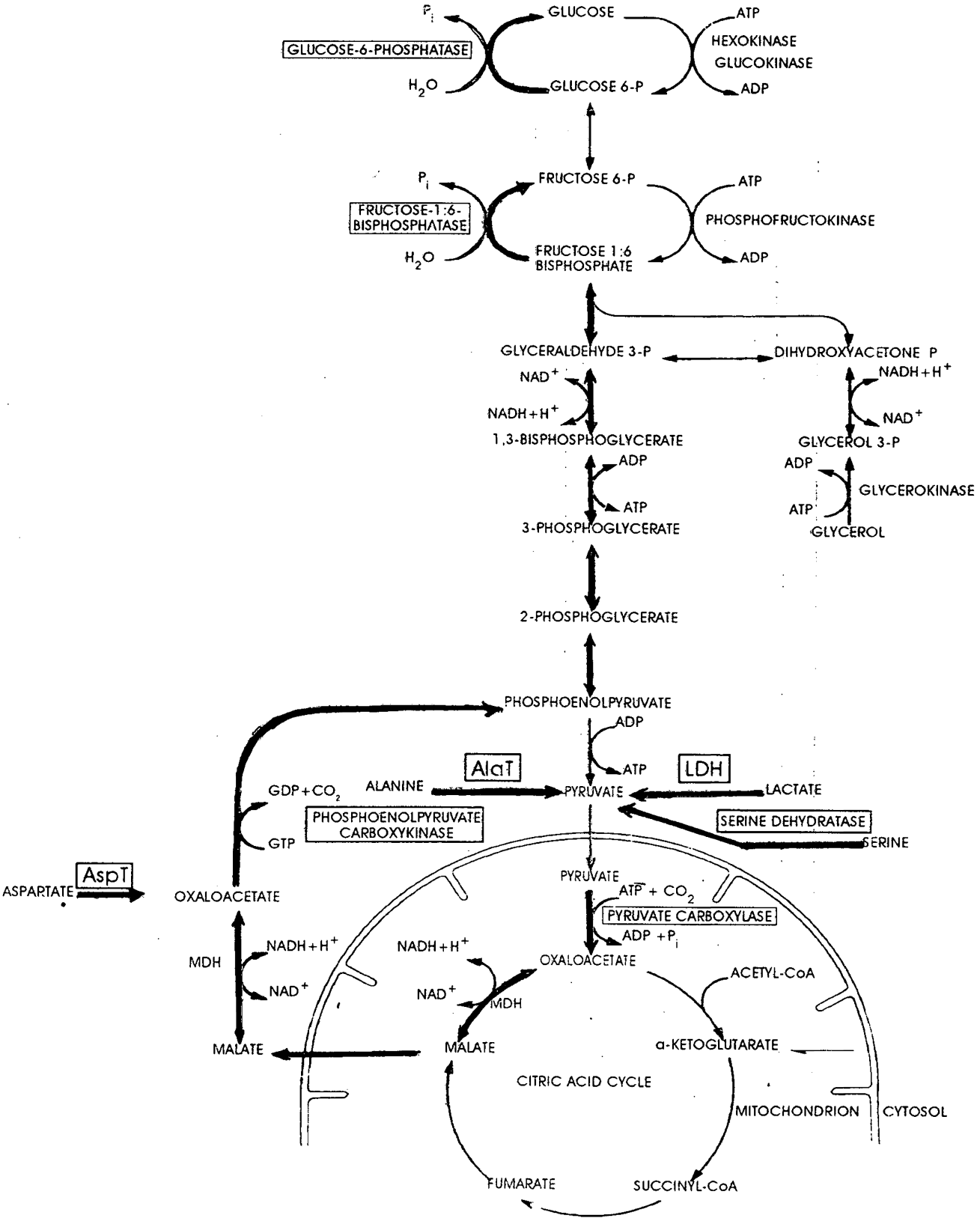


Fig. 4 Gluconeogenesis in rat liver.

3-phosphate dehydrogenase which converts 3-phospho-glyceroyl-phosphate to glyceraldehyde-3-phosphate. With lactate as the precussor of gluconeogenesis, NADH formed during conversion of lactate to pyruvate by lactate dehydrogenase is utilized, while with pyruvate as precursor of gluconeogenesis, NADH formed with reducing equivalents from mitochondria through oxaloacetate-malate shuttle is utilized[75, 76].

Hormonal control of gluconeogenesis: This can be divided into three categories,

1) Regulation of substrate supply. All gluconeogeneic substrates reach the liver in sub-saturating concentration. Thus regulation of substrate release into the blood from the extra hepatic tissues directly affects hepatic glucose formation.

2) The second category deals with the very important but relatively slow adaptive changes in enzyme activity due to regulation of protein synthesis and/or degradation.

3) The third category is concerned with the minute-to-minute regulation of gluconeogenesis by glucagon, catecholamines and insulin[73].

Gluconeogenesis comprises of 3 sets of enzymes.

1) Glycolytic enzymes with bidirectional activity:- These enzymes are common to glycolysis pathway as well as gluconeogeneic pathway.

2) The typical gluconeogeneic enzymes are pyruvate carboxylase, phospho-enol pyruvate carboxykinase, hexose di- phosphatase and glucose 6-phosphatase These enzymes catalyse reaction only in gluconeogeneic pathway at crucial points of glucose metabolism where they are opposed to glycolytic enzymes acting in one direction namely pyruvate kinase, phosphofructokinase, gluco-kinase or hexokinase.

3) A group of enzymes together with alanine transaminase aspartate transaminase, serine dehydratase and many other transaminases which are involved in the conversion of various amino-acids into it's gluconeogeneic precursors.

Aspartate transaminase :-

This enzyme also known as glutamic-aspartic transaminase plays an important role in gluconeogenesis. It converts the amino-acid aspartate to yield oxaloacetate which can be used up by gluconeogeneic enzyme to form glucose. It's mitochondrial fraction plays an important role in converting the oxaloacetate formed by the enzyme pyruvate carboxylase to aspartate thus facilitating it's transfer across the

mitochondrial membrane and then converting it again to oxaloacetate by the enzyme aspartate transaminase of cystosolic origin so the oxaloacetate (OAA) can be used by the enzyme PEPCK to form phospho enol-pyruvate. The dual (mitochondrial and cytosolic) intracellular distribution of malate dehydrogenase (MDH) and aspartate transaminase (aspT) is well established. Some data suggests that only the cytosolic fraction of aspT increases in condition of enhanced gluconeogenesis[51]. Both the anionic and cationic fraction of aspT are inhibited by glyceraldehyde 3-phosphate and it's naturally occurring homologues erythrose 4-phosphate and glycoldehyde phosphate, which therefore might participate in the regulation of gluconeogenesis[77]. Maleate and succinate are the most effective inhibitors of mitochondrial transaminase, whereas succinate, maleate, glutarate and adipate inhibit the supernatent isozyme.[78]

It is to be pointed out that when the mitochondrial OAA moves to cytosol via malate, reducing equivalents are transferred from mitochondria to the cytosol along with the 4 carbon precursor, while when mitochondrial OAA moves to the cytosol via aspartate, only the 4-carbon precursor is transferred[51]. Thus the former route (OAA-malate shuttle) might predominate during gluconeogenesis from pyruvate, while the latter (OAA-aspartate shuttle) might be of greatest importance, when lactate is the gluconeogeneic precursor, since in the latter instance reducing equivalents (NADH) are formed during the conversion of lactate to pyruvate[79]. Each of these 2 shuttles was

suggested to be working close to or at its maximal capacity and therefore transfer process between mitochondria and cytoplasm must be considered as potential rate-limiting and regulatory site for gluconeogenesis[79].

Alanine transaminase :-

Also termed as glutamic-pyruvic transaminase this enzyme catalyses the reaction between α-keto glutarate and L-alanine to give L-glutamic acid and pyruvic acid. Together with glutamic-aspartic transaminase, they play an important role in the transamination reaction leading to the elimination of nitrogen from amino acids. They are an important source of keto acids for Kreb's cycle and for gluconeogenesis alanine transaminase (ala T) predominates in the cytoplasmic compartment in the rat liver[80, 81]. It has been observed that the mitochondrial fraction of this enzyme is present in organs that can utilize alanine for glucose production (rat liver, pig liver and kidney) and is absent from rat kidney which cannot convert alanine to glucose. The cytosolic fraction of enzyme predominates in glycolytic tissues (muscles) and is involved in the formation of alanine from pyruvate[82]. Alanine and glutamine are most important since they are released in the greatest amounts from muscle. The gut[41, 83] takes up glutamine coming from muscle and releases alanine which is a predominant gluconeogeneic precursor which is why alanine accumulates in blood much more than other amino-acids[84].

Alanine is found to stimulate renal gluconeogenesis from a variety of substances probably by inhibiting the activity of the enzyme pyruvate kinase[51]. Various conditions such as high protein intake, diabetes, prolonged fasting and corticoid treatment produce a marked increase in liver ala T[85]. The level of ala T rises 5-fold under the influence of prednisolone at a time when the rate of enzyme destruction is increased approximately 3-fold, the increased ala T activity would thus have to result from a rather enormous increase in the rate of enzyme synthesis[86]. The rate of synthesis of ala T was changed 2-fold after 6.5 hours of prednisolone or 5 day of hydro-cortisone treatment. Tyrosine amino transferase (Tyr T) and tryptophan oxygenase activities in comparison, reach a maximum within a few hours after gluco-corticoid administration. The observation indicates that the stimulatory effects of gluco-corticoid hormone on the synthesis of liver proteins are sequential rather than simultaneous[85].

Serine dehydratase :-

It catalyses the conversion of L-serine to pyruvate and ammonia. Liver serine dehydratase is inversely related to the body size of species in higher animals. Specific activities of the enzyme are in the order: common shrew > mouse > rat > guinea pig > rabbit > calf, sheep, pig. In rat high activity is found in the liver and slight activity in the kidney. The liver enzyme is a cystosolic enzyme. It is induced by dietary serine or during an increased state of gluconeogenesis, resulting from such causes as starvation, feeding a

high protein diet, diabetes and hormone administration. The enzyme appears to require pyridoxal 5-phosphate for it's maximum activity. P-cholromercuribenzoate, elemental sulphur, L-cysteine, L-homo-cysteine inhibits the enzyme activity[87].

The enzyme is specific for L-serine and it does not catalyze the cleavage of L-homocysteine, cysteine, L-cysteine, DL-allocystathionine and L-homoserine. In neonatal rat liver, the enzyme serine-pyruvate aminotransferase predominates[87]. It converts the amino-acids serine to hydroxy pyruvate, which gets converted to glycerate and then to 2-phosphoglycerate which is then converted to glucose by reversal of glycolysis[88]. In adult liver[89] and in the fasted state[90] the serine is deaminated and cleaved hydrolytically to yield pyruvate by the enzyme serine dehydratase.

Phosphoenolpyruvate carboxykinase (PEPCK) :-

This enzyme affects the conversion of oxaloacetate to phospho-enol pyruvate in the presence of GTP or ITP[91], thus playing a fundamental role in gluconeogenesis[51] together with pyruvate carboxylase.

The intracellular localization of hepatic PEPCK (GTP) varies with species. In fact this enzyme is located primarily in the cytosol fraction in the rat, mouse or hamster[92], but has dual (cytosolic and mitochondrial) localization in cow, guinea-pig[51, 93] dog[51] and[94]

man and is primarily mitochondrial in pigeon and chicken. Thus PEPCK has shown the largest species difference. In rat liver PEPCK (GTP) is lower than pyruvate carboxylase[51], a fact that further indicates the importance of the former enzyme for gluconeogenesis. The key gluconeogeneic role played by PEPCK (GTP) is also suggested by the appearance of this enzyme during development, at the exact hour, when the hepatic gluconeogenesis becomes manifest[51]. PEPCK competes with citrate synthase enzyme for their common substrate OAA.

In perfused rat liver, the rate of conversion of oxaloacetate to phospho-enol pyruvate is 3 fold greater than the rate of conversion to citrate[95]. pyruvate carboxylase is primarily located in the mitochondria while PEPCK is primarily located in the cytosol (with respect to rats). Hence the oxaloacetate formed intramitochondrially by the pyruvate carboxylase must cross the mitochondrial membrane for being converted to phospho-enol pyruvate in the cytosol by the latter enzyme. Since the mitochondrial membrane is relatively impermeable to oxaloacetate, this compound may be converted to malate or aspartate in order to traverse the mitochondrial membrane[96]. In the cytosol, malate and aspartate can be reconverted to oxaloacetate, which through the action of PEPCK may enter the gluconeogeneic sequence[96]. These substrate interconversions are effected by the mitochondrial and cytosolic fractions of malate dehydrogenase and asp. T.[96, 97], which can then

be used by enzyme PEPCK to synthesize phospho-enol pyruvate, which through reversal of glycolysis would yield glucose.

PEPCK is present in significant amounts in rat muscle and heart. However, owing to the virtual absence of the key gluconeogeneic enzyme pyruvate carboxylase, gluconeogenesis has a negligible role if any in muscle. The subcellular distribution of PEP-CK in kidney of various species resembles that in the liver[98].

Regulation by Hormonal Factors :-

Catecholamines :-

Catecholamines stimulate gluconeogenesis from lactate in rat liver and this effect might be mediated by cAMP[99]. Catecholamines also stimulate gluconeogenesis from dihydroxy acetone, although this stimulation is smaller than that caused by glucagon. Therefore sympathetic nervous activity leading to the intra hepatic release of catecholamine, may play a role in increasing gluconeogenesis and lactate uptake especially during exercise and hypoglycaemia[99]. Recent data suggest that catecholamines stimulate gluconeogenesis from lactate, pyruvate or alanine by a mechanism apparently unrelated to cAMP[22, 100]. Moreover it has been observed that the alpha-or beta-adrenergic blocking agents, together with epinephrine did not cause any significant change in gluconeogenesis as compared to epinephrine alone[51]. PEPCK of rat liver is stimulated by

catecholamines, a change prevented by actinomycin D[101]. Intra-peritoneal injection of DB-cAMP resulted in a 4 to 5 fold induction of tyrosine aminotransferase (Tyr T) reaching a peak at 2 hours and values were still significantly higher than normal at the end of 5 hours.[102]

Insulin :-

Insulin (and proinsulin) inhibits gluconeogenesis in perfused rat liver[51] and antagonizes the effect of epinephrine and glucagon on glucose out put by the perfused liver[103]. Basal insulin in the over-night-fasted dog inhibits glucose formation from alanine; this process, however, is stimulated by glucagon which suggests that the effect of the latter hormone prevails during insulin deficiency[104]. Insulin prevents the increase in the synthesis of PEPCK (GTP) produced by either DB-cAMP or dexamethasone and this antagonism is concentration dependent which does not require the simultaneous presence of glucose[105]. Insulin regulates the enzyme at the translational or post-translational levels[105]. Insulin prevents the elevation of serine dehydratase in normal and diabetic rat liver[106]. Insulin treatment in diabetic humans was found to decrease the hepatic enzyme asp T[107]. It thus appears that insulin is a suppressor of key gluconeogeneic enzymes[108, 109]. Besides affecting hepatic gluconeogeneic enzymes, insulin decreases the flow of gluconeoegeneic substrates from peripheral tissues to liver, in as much

as the hormone favor the oxidation of pyruvate in the tissue[110] and diminishes the release of amino-acids (alanine) from muscle. In kidney of rats phosphofructo kinase and pyruvate kinase are rapidly increased by intravenous injection of insulin[111], while hexose di-phosphatase is simultaneously reduced.

Effect of dietary carbohydrates :-

Carbohydrate intake depresses key gluconeogeneic enzymes. Continuous infusion of glucose to normal rats causes a decrease in hepatic PEPCK (GTP) and serine dehydratase activities[112]. Dietary carbohydrates also depress hepatic aspT, alaT. [51] and TyrT. [51]. Constant infusion of fructose to perfused rat liver caused a decrease in PEPCK (GTP), pyruvate carboxylase and serine dehydratase activities[112]. Perfusion of rat liver with lactate (18 mM) causes induction of both pyruvate carboxylase and PEP-CK (GTP)[112]. Feeding with lactate or glycerol inhibits the increase in serine dehydratase produced by starvation[113]. Glycerol feeding also inhibits the increase in hepatic PEPCK (GTP) and Tyr T. occurring with starvation[113].

Effect of dietary protein and amino- acids :-

PEP-CK (GTP) is increased in rat liver by high protein diet, while pyruvate carboxylase is not[51]. Low protein diet causes a decrease in Tyr T.[51, 114], and ala T.[114, 115] in total liver

homogenate. Ala T. also decreases in liver cytoplasmic fraction. Serine dehydratase declines in liver in low diet intake[116, 117]. Threonine dehydratase also decreases in rat liver[118]. High protein diet causes an increase in rat liver asp T, ala T. and serine dehydratase[118]. Amino-acid mixture administration also increases rat liver serine dehydratase[119]. Glycine, tryptophan, methionine, valine, threonine causes an increase in rat liver serine dehydratase[51,119]. Excessive intake of threonine causes a reduction in rat liver asp T.[51]. In the heart of protein depleted rats a low activity of aldolase, lactate dehydrogenase[51] as well as asp T, ala T. is observed[51].

Diabetes mellitus :-

Diabetes mellitus is a common disease of insulin and glucose metabolism characterized by either destruction of the pancreatic β islets due to an autoimmune process (Type I) or decreased insulin action combine with inadequate release of insulin from the pancreas (Type II). Patients with Type I diabetes need exogenous insulin to prevent diabetic ketoacidosis and control their hyperglycaemia. On the other hand, most patients with Type II diabetes can be treated without exogenous insulin. Type II diabetes is characterized by insulin resistance and often occurs in obese persons though some require an oral hypoglycaemic agent or insulin to control their hyperglycaemia[120]. The chronic sequelae of diabetes includes retinopathy, neuropathy, nephropathy and premature atherosclerosis. Evidence accumulated from

biochemical and animal experiments suggest that chronic hyperglycaemia is an important factor in the development of most of the long term diabetic complications[121, 122].

Experimental diabetes :-

It can be achieved by pancreatectomy or by administration of β cyto-toxic agents such as alloxan, streptozotocin[123, 124] and bromocriptine[125]. These agents destroy the insulin secreting β cells of the pancreas.

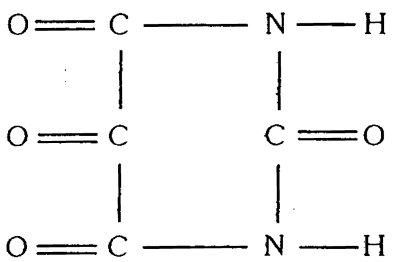
Alloxan :-

Alloxan (fig. 5a) is a 2,4,5,6-tetra oxo hexa hydro pyrimidine having a structure similar to that of uric acid.

Alloxan by series of redox reactions generates superoxide radicals which causes lipid peroxidation and single strand DNA breaks. The ensuing DNA repairs deplete NAD^+ essential for proinsulin synthesis and other physiological processes. This result in oxidative damage and tissue necrosis and therefore diabetes mellitus[126].

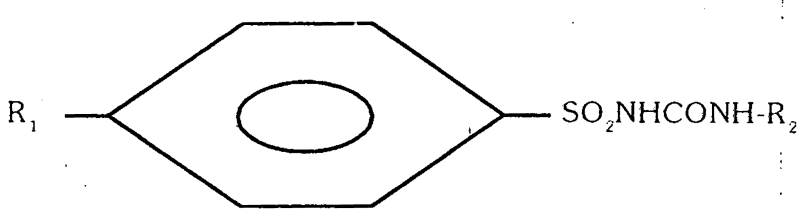
Biological effects :-

The β-cell toxicity of alloxan was discovered in 1943, while testing the nephrotoxicity of uric acid derivatives in rats and rabbits[127]. The animals which received alloxan showed the classical signs of

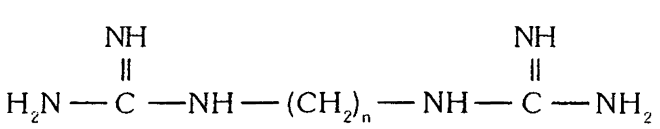


Alloxan

Fig. 5a :- Diabetogenic Agent



Sulphonylurea



Diguanidine

Fig. 5b:- Oral hypoglycaemic agents

human diabetes i.e. marked hyperglycaemia, glycosuria, polydipsia and polyuria, loss in bodyweight despite polyphagia, hyper lipaemia, ketonuria and acidosis. Partial or total necrosis of pancreatic islets were observed; other tissues except kidney showed no damage[123]. In the pancreas, exocrine cells, endocrine cells other than β cells and connective tissue were unaffected by alloxan[128, 129]. It was also shown by autoradiography that mouse pancreatic β cells accumulate alloxan[130]. Thus alloxan is a β-cytotoxin with an immediate toxic effect on the β cells of islet of langerhans, leading to chronic experimental or chemical insulin dependent diabetes mellitus[123].

In normal non-fasted mice, a triphasic blood sugar curve develops after a diabetogenic dose of alloxan. The initial marked hyperglycaemia, probably due to liver glycogenolysis last for a period of 1 to 4 hours after alloxan administration. This is followed by severe hypoglycaemia within 6 hours upto 12 hours due to outpouring of insulin from damaged cells, often resulting in convulsion and death. Chronic long lasting hyperglycaemia results in most cases. In fasted mice, the early hyperglycaemia is severely attenuated, while the hypoglycaemia is more severe and the diabetic phase manifests itself within 24-48 hours[123, 124].

Oral hypoglycaemic agents :-

In most patients with mild NIDDM, all that is required to control their disease is an exercise program and a diet that results in

weight loss. However some patients are not able to achieve satisfactory control of their diabetes with diet and exercise alone. This group may benefit from the use of a oral hypoglycaemic agents.

Two classes of oral hypoglycaemic agents: the sulphonylurea and the biguanides (fig. 5b) are in use today.

Sulphonylurea :-

The first-generation sulphonylureas include tolbutamide, chlorpropamide while the second generation sulphonyl urea includes glyburide (micronase and diabeta) and glipizide (glucotrol). Sulphonylurea improves diabetic control by two mechanisms: they increase insulin secretion and they improve insulin action of target cells[131].

Mechanism of action :-

To work, sulphonylurea require endogenous synthesis of insulin by the β cells. Sulphonyl ureas exert their effect by enhancing the secretion of preformed insulin. Binding of a sulphonylurea to a receptor on the β-cell membrane stimulates the release of insulin in the absence of other secretagogues [132]. Sulphonylurea also potentiates glucose mediated release of insulin. All sulphonylureas have same mechanism of action but differ in potency based on their affinity to the sulphonylurea receptor[133]. The second generation sulphonylureas, glipizide and glyburide bind more tightly to the

receptors[133], than do the first generation and therefore are equally effective at lower dosages[120]. Long term treatment with sulphonylurea improves tissue sensitivity to insulin and possibly increases insulin-receptor binding[134]. Sulphonylurea therapy enhances the ability of insulin to inhibit production of glucose by the liver and to stimulate utilization of glucose. This improvement in hepatic and muscle cell sensitivity to insulin in patients taking sulphonylurea also may be partly explained by the effect of the drug to reduce the hyperglycaemic threshold for insulin secretion[135].

Biguanides :-

The 2 drugs from this biguanides groups are phenformin and metformin.

Mechanism of action :-

Metformin decreases insulin resistance without directly affecting the insulin secretion. Its most important action may be to reduce hepatic glucose overproduction, probably by inhibiting gluconeogenesis. Other proposed mechanism of action includes direct stimulation of glycolysis in peripheral tissues, slowing of glucose absorption from the gastro-intestinal tract and increased insulin binding to insulin receptors.

Other hypoglycaemic agents :-

In the recent past, studies have been carried out on the hypoglycaemic activity of *Peuraria tuberosa*[136] and *Hamiltonia suaveolens*[137] among various other plant extracts. Methanolic extracts of *Peuraria tuberosa* caused at concentration of 50 mg/kg b.wt a maximum hypogl caemia (35.2% lowering) at the end of 5 hours. It's effect lasted beyond 24 hours. Crude ethanolic extract of *Hamiltonia suaveolens* showed a maximum hypoglycaemia (45% lowering) at a dose level of 500 mg/kg b.wt in alloxan diabetic mice at the end of 5 hours.

In this context, it is to be mentioned that vaccine or cell-extract of *Bordetella pertussis* was reported to cause hypoglycaemia [138] as well as significant increase glucose tolerance in experimental animals as has been confirmed by studies in our laboratory[139].

Bordetella pertussis extract

The organism :-

The genus is named after Jules Bordet, who with O.Gengou first isolated the organism causing whooping cough. The other two species are *B.bronchisepta* and *B. parapertussis* [140].

Bordetella pertussis is strictly aerobic, minute cocco-bacilli, (0.2-0.5 μm by 0.5-2.0 μm), occurring singly or in pairs or

occasionally in short chains. It is non-motile, Gram-negative and often bipolar stained. Capsules may be demonstrated in virulent strains[140].

Colonies on Bordet-Gengou agar are smooth, convex, pearly, glistening, nearly transparent and surrounded by a zone of hemolysis without definite periphery. The optimum temperature for growth is 35-37°C. Metabolism is respiratory and never fermentative. It is chemo-organotrophic, requiring nicotinamide, organic sulfur (e.g cysteine) and organic nitrogen (amino acids). It oxidatively utilizes glutamic acid, proline, alanine, aspartic acid and serine, with the production of NH_3 and CO_2[140].

B. pertussis is a mammalian parasite and pathogen, localizing and multiplying among the epithelial cilia of the respiratory tract. This results in whooping cough, an acute infectious disease of trachea and bronchi, lasting 1-2 months with typical violent and paroxysmal cough. Children under the age of 7, especially under 2, are susceptible to the disease[141].

Phase variation :-

On the basis of serological reaction *B. pertussis* shows four well-marked agglutinable phases - I, II, III and IV. Phase I or freshly isolated strains are encapsulated, virulent, hemolytic, and require the presence of X and V factors of blood for growth. Only the toxic

phase I cells are suitable for vaccine production to provide immunity against pertussis. Phase IV cells, non-protective and non-toxic, grow on nutrient agar and lack multiple virulent factors. Phase I colonies are smooth, which under artificial cultivation or sub-culturing gradually change to rough colonies of phase II, III, IV. However, addition of sufficient fresh blood to the medium makes it possible for the parasitic phase I to persist unaltered for long periods. Phase II and III are only partially rough and can revert to I or irreversibly change to IV[142].

Phase I cells grown in high Mg^{2+}, low Na^{+} medium show loss in certain envelope proteins, intracerebral protective activity, pertussis toxin, heat labile toxin (56°C), adenylate cyclase activity and adjuvanticity. The concomitant losses suggest that i) a single site of action is controlled by one enzyme. ii) the enzyme affects the fastidiousness of growth of phase I *B. pertussis* cells. iii) the enzyme is lost during mutation to phase IV[140].

Virulence factors :-

B. Pertussis cells have an array of biologically important virulence factors that individually or collectively play an important role in host-parasite relationship [143, 144]. These factors includes : adenylate cyclase toxin (AC), agglutinogens, dermonecrotic toxin (DNT) or heat labile toxin (HLT) or lethal toxin, filamentous or fimbrial

hemagglutinin (FHA), lipopoly saccharide (LPS) endo toxin, Pertussis toxin (PTx), tracheal cytotoxin (TCT).

Pertussis Toxin :-

It has also been called by other names on the basis of its activities such as lymphocytosis or leucocytosis promoting factor (LPF), LPF-Hemagglutinin (LPF-HA), histamine sensitizing factor (HSF) and Islet-Activating Protein (IAP). Besides increasing the antibody response in mice to antigens, it acclerates experimental allergic encephalomyelitis in lewis rats, increases the production of Ig E (immunoglobulin E) antibodies and protects mice from intracerebal challenge with *B. pertussis* cells[145]. Mouse protective antigen was isolated in 1981 and given the unifying name pertussigen[146]. On characterization, the crystalline pertussigen was found to exert all of the above activities tested[147]. PTx has been isolated from *B. pertussis* cell membrane[145] or supernatent of cell-cultures[144] PTx (MW 90-107 KD) is a holotoxin based on A-B model with 5 dissimilar sub-units. The A protomer (sub-unit S1) has ADP-ribosyl transferase activity while β oligomer (sub units S2, S3, S4, S5) mediates binding of toxins to target cell[148]. The A protomer catalysis the transfer of an ADP-ribosyl moiety from NAD to one of the guanine nucleotide regulatory components in the receptor adenylate cyclase system[149].

Review of literature on the effect of *B. pertussis* extract :-

The hypoglycaemic effect in infants during *pertussis* infection or following pertussis vaccination is well documented[150, 151, 152]. *Pertussis* vaccine has been reported to improve blood glucose regulation in some diabetic patients[152]. In another study, pertussis vaccine was found to cause an insulin like blood sugar lowering in rats[153]. Pertussis vaccine prepared from cells of strain no. 18-323 produced a marked hyperinsulinemia in mice with a modest hypoglycaemia (15-20%)[154]. The vaccine given before alloxan also attenuated alloxan induced hyperglycaemia[153], though is less effective, if given after alloxan administration. Munoz and Bergman later isolated an active substance called pertussigen from *B. pertussis* cells which induce hypoglycaemia in mice[145]. They extracted pertussigen from the cell-membrane[155] and also the culture supernatent[146]. These proteins have also been isolated and characterized as islet activating protein (IAP) by Yajima [156] from the culture supernatent of *B. pertussis* phase I cells.

IAP doubled the insulin secretory response to glucose loading 3-8 days later[156, 157, 158, 159]. This may be mediated by a modification of the β adr receptors in the endocrine pancreas by IAP or by enhanced glucose utilization peripherally[160]. IAP may also cause activation of the native calcium ionophores on the islet cell-

membrane thus rendering more calcium available to the stimulus-secretion coupling mechanism of insulin secretion[161]. Mice infected intranasally with *B. pertussis* (strain no. 18-323) cells showed severe hypoglycaemia secondary to hyper insulinemia. PTx also caused the same effect at a dose of 150 ng per mouse when injected intravenously[162]. M. Kishore have also isolated a protein from B. pertussis cell-membrane (strain no 25525) which produce highly significant lowering of blood sugar in mice[139]. Preliminary studies by P. Ramakrishnan and Anita Kala reported that the extract from B. pertussis cells (strain no. 25525) loweredblood sugar to hypoglycaemic levels (45 % lowering) at the dose level of 1 mg/kg b.wt. at the end of 72 hours[163, 164] together with significant increase in G6PD levels indiabetic animals. Subsequently using the challenge strain no 18-323 in our laboratory significant hypoglycaemia was observed at the end of 12 hours. It also significantly elevated the activities of glycolytic enzymes in mice liver[165].

The *B. pertussis* extract reversed the diabetogenic effect of alloxan by increasing the rate of utilization of glucose and by restoring the antioxidant enzymes to their normal values which were otherwise depleted by alloxan[166]. Lipid peroxidation was also significantly reduced[166]. It was found to significantly restore the levels of glucose 6-phosphate dehydrogenase (G6PD), the rate limiting enzyme of the pentose cycle in the liver of diabetic mice[164].

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The effect of B. pertussis extract has been studied on glycolytic enzymes and also anti oxidant enzymes. It's blood sugar lowering effect has been compared with some known oral hypoglycaemic agents.

However the effects of *B. pertussis* on vital physiological mechanisms such as glycogenolysis and gluconeogenesis are not known. Since epinephrine is known to stimulate both glycogenolysis and gluconeogenesis it was decided to study the effect of *B. pertussis* extract on the metabolic changes induced by epinephrine.

Aims and Objectives

To study the effect of *B. pertussis extract* in epinephrine treated mice and elucidate it's mechanism of action. This required the assessment of two mechanisms, namely glycogenolysis and gluconeogenesis in the liver, apart from estimation of blood sugar and serum insulin levels.

IN LIVER :-

I (1) **Glycogenolysis** :- It is a cAMP mediated process, which through activation of enzyme cascade leads to formation of glucose. The parameters to be studied include

a) **Glycogen** :- which is the main storage polysaccharide in the animal cell.

b) ***Phosphorylase a;*** which catalyses the breakdown of glycogen to glucose 1-phosphate from which glucose 6-phosphate and then free glucose is formed.

$$(\alpha\text{-}1,\ 4\text{-Glucosyl})_n + Pi \xrightarrow{\textit{Phosphorylase a}} (\alpha\text{-}1,\ 4\text{-Glucosyl})_{n-1} + \alpha\text{-D-Glucose-1-P}$$

(Glucose 1-phosphate)

(2) **Gluconeogenesis** :- This process involves formation of glucose from non-carbohydrate sources. The enzymes to be studied include.

a) **Serine dehydratase** :- It is a pyridoxal phosphate containing enzyme which converts the amino acid L-serine

through elimination of water and hydrolytic loss of ammonia to yield pyruvate.

L-Serine $\xrightarrow[\text{(Pyridoxal phosphate)}]{\text{Serine dehydratase}}$ pyruvate + H_2O + NH_4

(b) **Aspartate transaminase (aspT)** :- It is a gluconeogeneic enzyme which converts the amino-acid, L-aspartate and α-keto glutarate to yield oxaloacetate which can be converted to glucose through reversal of glycolysis.

L-Aspartic acid + α keto glutarate $\underset{\text{Pyridoxal phosphate}}{\overset{\text{aspT}}{\rightleftharpoons}}$ oxaloacetate + L-glutamate

c) **Alanine transaminase (alaT)** :- It is a gluconeogeneic enzyme which converts the amino acid L-alanine and α-ketoglutarate to yield pyruvate.

L-Alanine + α ketoglutarate $\underset{\text{Pyridoxal phosphate}}{\overset{\text{alaT}}{\rightleftharpoons}}$ pyruvate + L-glutamate

d) **Phosphoenolpyruvate carboxykinase (PEP-CK)** :- This is the rate determining enzyme in gluconeogenesis. It converts the metabolite oxaloacetate to phosphoenolpyruvate.

Oxaloacetate + GTP $\xrightarrow[Mg^{2+}]{\text{PEPCK}}$ phosphoenolpyruvate + CO_2 + GDP

All these parameters will be assayed in mice liver. The levels will be studied in epinephrine treated mice as well as in animals pretreated with *B. pertussis* extract.

The enzyme levels will also be studied in alloxan diabetic mice and in alloxan diabetic mice treated with *B. pertussis* extract. This study will be done in order to examine whether the *B. pertussis* extract has any independent action in diabetic animals.

II **To estimate Blood sugar levels and serum insulin levels** :
As epinephrine is known to cause hyperglycaemia and *B. pertussis* extract to lower blood sugar levels, it was thought to estimate blood sugar and insulin levels in different groups.

III To explain the reversal of the above mechanisms by *B. pertussis* extract.

Materials and Methods

1. Chemicals :-

All chemicals are of analytical grade, unless mentioned otherwise. The enzymes were purchased from sigma chemical company, St. Louis, MO., U.S.A. and Boehringer Mannheim.

2. Cell cultivation, protein extraction and partial purification :-

"*Bordetella pertussis*" cells (challenge strain no. 18-323) were obtained in a lyophilized form from Haffkine Biopharmaceutical corporation Ltd, Bombay. Cultivation was done in strictly sterile conditions using the facilities of Bacteriology Department of Haffkine Institute, Bombay.

2.1 Preparations for cultivation :- All glassware were thoroughly washed rinsed with distilled water (D/W), dried and heat sterilized at 180°c for 60 minutes or autoclaved at 121°c for 20 minutes.

2.2 Preparation of Verwey's Liquid Medium (VLM)[167]:-

Solution A, B, C were prepared as follows:

A)	i) Casamino acids (Difco, Technical grade)	140.0 gms
	ii) NaCl	30.0 gms
	iii) KCl	2.5 gms
	iv) $MgCl_2.6H_2O$	1.0gms
	v) KH_2PO_4	5.0 gms

The above components were dissolved in 1 litre hot distilled water and brought to a boil.

B) Soluble starch 10.0 gms

To a slurry in 50 ml distilled water, 200 ml boiling distilled water was added. Boiling was continued till the starch was dissolved.

C) i) glutathione (reduced) 0.1 gm

ii) nicotinic acid 0.2 gm

L-Cystine may be used, instead of glutathione in the same amount. The above components were dissolved in 250 ml hot distilled water.

The solutions A, B and C were mixed. About 1.5 litres cold distilled water was added to get it to room-temperature. The PH was adjusted to 7.2 with 10 N NaOH and the volume was made to 10 litres. The medium was distributed as follows: 2.0 litres in a 5 litre round bottom flask, 50 ml in 250 ml conical flasks, 5 ml in 18 ml test-tubes, plugged with non-absorbent cotton. The 2.0 litre media were autoclaved at 121°c for 35 minutes and .smaller amount of media at 121°c for 20 minutes.

2.3 Preparation of Bordet-Gengou blood agar (BGBA) :-

4.0 gms of dehydrated Bordet - Gengou agar base (*Hi-media laboratories*) was dissolved in 100 ml of hot distilled water containing 1.0 ml glycerol. This was boiled to dissolve completely and autoclaved at 121°c for 15 minutes.

Completed medium[140]:- 15-20 ml fresh sheep, rabbit or horse blood was collected and added to a sterilized flask containing 5-6 glass beads, under aseptic conditions. The flask was plugged and swirled for 10 minutes. The defibrinated blood was then added under aseptic conditions to the melted agar maintained at 45°c. The agar was thoroughly mixed and 5 ml aliquots were dispensed into sterile test-tubes and left at an angle of 45°c to solidify. It was incubated at 37°c for a preliminary sterility check. The slants were then packed and stored at 4°c before use. Satisfactory slants were cherry red in colour and moist at the time of use.

2.4 Cultivation methodology :- The lyophilized cells were reconstituted in about 0.5 ml of Sterile VLM and incubated in 2 ml of Sterile VLM at 35°c for 30 minutes in the shaker. The revived culture was then innoculated in sterile slants of BGBA and incubated at 35°c for 48 hours. This was checked for the typical colonies of "*B. pertussis*" cells. Some of this primary culture was lyophilized and stored at 4°c. The slant was washed with sterile VLM and the culture was innoculated in 5 ml of sterile VLM. This was incubated at 35°c for 24 hours in a rotary shaker. The culture was then transferred to 50 ml sterile VLM and incubated as before for 48 hours. The seed culture thus developed was checked for purity. If pure, it was innoculated into 2.0 litres of sterile VLM and incubated on the shaker at 35°c for 48 hours. Characteristically, no pellicle was formed. A 0.5 ml sample of the culture was removed for purity and phase variation tests, the rest of the culture was inactivated by adding 5.75 ml of 40%

formaldehyde per litre of culture and kept at room-temperature for 120 hours.

i) <u>Purity tests</u> :-

a) A drop of culture was innoculated on sterile nutrient agar and incubated at 35°c for 48 hours. Absence of growth confirmed purity of culture.[140] (Nutrient agar :- peptone 1 gm, NaCl 0.5 gm, Beef extract 0.3 gm were added to 100 ml distilled water. The solution was warmed to dissolve and then cooled. PH was adjusted to 7.4 with 1 N NaOH. 2 gms of agar was then added and the solution boiled. The 5.0 ml of clear solution was dispensed into each tube, plugged and then autoclaved at 121°c for 20 minutes. The tubes were then kept at an angle of 45°c to solidify and sterility check was done at 37°c for 24 hours).

b) A loopful of the culture was smeared on a glass slide, air-dried and heat fixed, this was stained by Gram's method[168]. The slide was flooded with crystal violet (0.1%) for 1 minute. It was then washed with water and flooded with Gram's Iodine (0.1%) for 1 minute. It was drained and washed with alcohol till just a violet colour was retained. This was stained with Gram's Safranine for 45 seconds. It was again washed and blot dried. The smear was observed microscopically under oil immersion

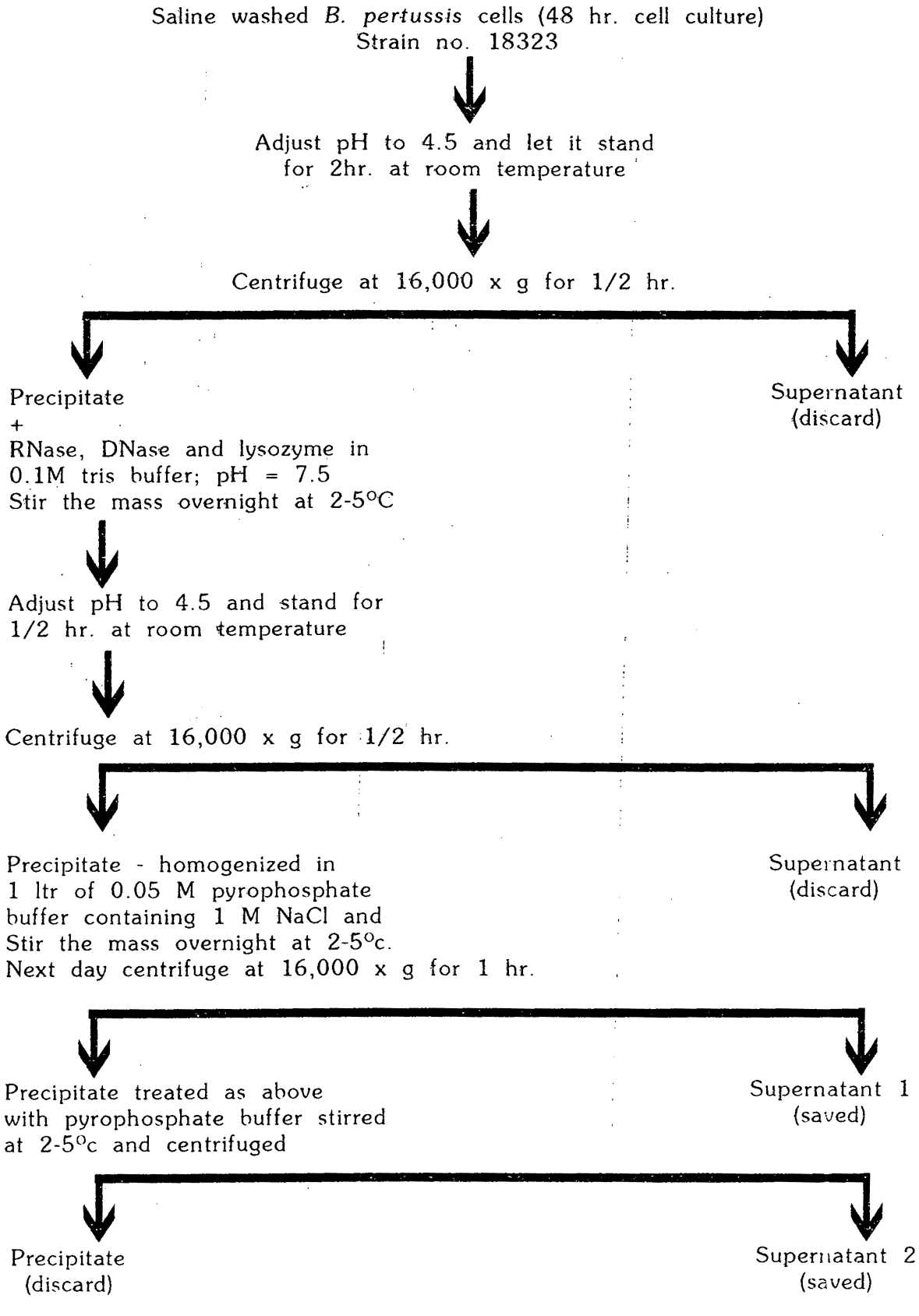
lens (100 x magnification). "*B. pertussis*" cells appear as pink (gram-negative) short rods. (cocco bacilli).

ii) Phase variation test [169]:- Phase variation was checked by innoculating a drop of culture on 10% horse serum agar slant (10% horse serum in nutrient agar base). These slants were incubated at 35°c for 48 hours. Absence of growth indicated that there was no phase variation[169].

2.5 Preparation and partial purification of "*B. pertussis*" extract :-

The procedure of Munoz and Bergman[155] was followed to obtain the biologically active protein from 4 litres of a 48 hours inactivated culture(Fig. 6). The culture was centrifuged at 16000 x g for 30 minutes in centrikon - T 324 centrifuge and the pellet washed twice with saline. The pellet was then dissolved in 80 ml saline. The PH was adjusted to 4.5 with concentrated HCl and it was kept at room-temperature for 2 hours. The cells were collected by centrifugation at 16000 x g for 30 minutes and the supernatent was discarded. 100 mg Lysozyme, 2.5 mg DNase and 2.5 mg RNase was dissolved in 1 litre 0.1 M Tris buffer of PH 7.5 and this was added to the pellet suspended in 1 litre of the same buffer. The solution was stirred for 4 hours at room-temperature and then overnight at 5°c. Concentrated HCl was then added to bring the PH to 4.5. After 30 minutes the precipitate formed was collected by centrifugation at 16000 x g for 30 minutes at 5°c. The precipitate was homogenized in 1 litre of 1 M NaCl,

Fig. 6. Flow Diagram of *B. pertussis* cell extraction



Supernatants 1 & 2 pooled, dialyzed and concentrated. Protein concentration determined by the method of lowry et al.

0.05 M sodium pyrophosphate buffer, PH 7.4 and stirred gently overnight at 5°c. This suspension was centrifuged at 16000 x g at 5°c for 1 hour. The supernatent obtained was saved and the pellet was rehomogenized with 500 ml of more buffer and stirred overnight at 5°c. Next day the suspension was centrifuged at 16,000 x g at 5°c for 1 hour. The second supernatent was pooled with the first supernatent. This extract was dialysed (dialysis bag mol. wt cut off value = 12-14KD) extensively for 3 days against two daily exchanges of distilled water. The extract was then concentrated. After estimating its protein concentration, it was aliquoted and stored at -20°c.

2.6. Estimation of the protein, carbohydrate and lipid content of the extract :-

2.6.1. Protein content was estimated by Folin -Lowry's method

Principle:

The peptide (-CO-NH-) bond of protein forms a purple complex with copper ions in an alkaline medium. Also the aromatic amino-acids tryptophan and tyrosine present in the protein reduce the phosphomolybdic acid and tungstate of the folin-ciocalteau reagent to give a dark blue complex. The blue colour obtained is directly proportional to the protein concentration. The method is 100 times sensitive than Biuret method.

Reagents:

i) **Alkaline $CuSO_4$ solution:**

a) 2% Na_2CO_3 in 0.1 N NaOH

b) 0.5% $CuSO_4$ $5H_2O$ in 1% Na K tartarate,

Alkaline $CuSO_4$ is prepared fresh before use by mixing 50 ml of 'a' with 1 ml 'b'

ii) **Folin - Ciocalteau reagent** :- This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids. The commercial 2N reagent is diluted 1:2 just before use.

iii) **Stock Standard** :- Bovine serum albumin (BSA) 100 mg% or 1 mg/ml in distilled water aliquots stored at -20°c.

iv) **Working Standard** :- This 100 µg/ml working standard was prepared by 1:10 dilution of stock standard.

Procedure :-

To plot the standard graph, additions were made in the range of 10 to 100 µg/ml from the working standard to make a volume of 1 ml. Test samples were also added in various dilution to make a final volume of 1 ml. To each tube 5 ml of alkaline CuSO4 was added. The tubes were mixed well and incubated at room-temperature for 15 minutes. 0.5 ml of diluted folin-ciocalteau reagent was added rapidly with immediate mixing. After 30 minutes incubation, the colour developed

was read against reagent blank at 660 nm. The concentration of the test sample was extrapolated from the standard graph.

2.6.2 Estimation of total carbohydrates by phenol-sulfuric acid method[171].

Principle :-

Simple sugars, oligosaccharides, polysaccharides and their derivatives including methyl esters or potentially free reducing groups give an orange yellow colour, when treated with phenol-sulfuric acid reagent. Since this follows the Beer-Lambert's law, the concentration of the unknown can be estimated.

Reagents :-

i) 5% W/V phenol in distilled water.

ii) concentrated H_2SO_4 (specific gravity 1.84)

iii) Stock standard:- Glucose solution 100 mg%

iv) Working Standard:- dilute stock standard 1:10

Procedure :-

Standard (20-100 µg/ml), distilled water (blank) and the test-sample of different dilution were added to make a final volume of 1 ml. Then 1 ml of 5% phenol was added to each tube and mixed. 5 ml of concentrated H_2SO_4 was added to the surface of the liquid and tube swirled for rapid mixing. The tubes were allowed to stand for 10 minutes.

The clear orange colour (stable for several hours) was read at 488 nm for hexoses and 480 nm for pentoses.

2.6.3 Total lipid estimation by the method of Fring's et. al[172].

Principle :-

Lipids react with vanillin in a medium of sulfuric acid, phosphoric acid to form a pink chromogen. This follows Beer-Lambert's law and can be used to estimate total lipid concentration in test-sample.

Reagents :-

i) Vanillin reagent :- Vanillin 0.6 gm% in distilled water. Stable for two months, when stored in a brown bottle at room-temperature.

ii) Phosphovanillin reagent :- To 35 ml of vanillin reagent, add 5 ml distilled water in a 250 ml Erlenmeyer flask. 60 ml of concentrated phosphoric acid was added to it with constant stirring. It was stable for 2 months when stored in brown bottle at, room-temperature.

iii) Standard :- Olive oil (100 mg) was dissolved and made to 100 ml with absolute alcohol. Solution was stable for 1 month at $4^{o}c$.

Procedure :-

20 µl distilled water (blank), the standard or sample (unknown) were added to 20 ml capacity test-tubes. Concentrated H_2SO_4

(0.2 ml) was added to each tube and was mixed well. The tubes were kept in boiling water for 10 minutes and were then cooled. 5.0 ml of phospho-vanillin reagent was then added, the tubes were mixed and incubated at 37°c / 15 minutes. Absorbance was measured at 540 nm against blank. Concentration of 200, 400, 600 800 and 1000 mg% were used to plot the standard curve.

3. Animals :-

Swiss, white male albino mice (24-26 gms body-weight) were supplied by HBPCL, Bombay. They were acclimatized to laboratory conditions and were given food and water *ad libitum*. They were fasted for 18 hours prior to each assay. To study the effect of *B. pertussis* extract (*B.P. ext.*) in epinephrine treated mice, the animals were divided into four groups :

I. **Normal saline control** :- 0.1 ml saline (0.154 M NaCl in distilled water) was injected intravenously and parameters assayed.

II. ***B. pertussis* extract group :-** It was given intravenously at the dose of 1 mg/kg body weight in terms of protein concentration and the parameters estimated at the end of 6 hours.

III. **Epinephrine treated group** :- Epinephrine (Adrenaline Bi Tartarate 1 mg/ml, Walker Pharmaceuticals Pune) was

given at the dose of 0.4 mg/kg bodyweight intra-peritoneally [173] and parameters estimated at the end of different time intervals.

IV. **B. pertussis + epinephrine group** :- *B.P. ext.* was administered in such a way that its maximum action (6 hours) coincided with the peak action of epinephrine, for the different parameters studied.

To study the independent effect of *B.P. ext*, the animals were divided into two groups.

V. **Alloxan (48 hours) group** :- Alloxan was injected intravenously at the lethal dose of 75 mg/kg body-weight[174] and parameters assayed at the end of 48 hours.

VI. **Alloxan + B.P. ext. group** :- The alloxan diabetic mice were injected with B.P. ext. at the end of 42 hours and assay was performed at the end of 48 hours.

4) Dose response and time rate studies :-

The optimum dose of epinephrine to be used for further studies and the duration of maximum hyperglycaemia were established. Similarly, the time and dose required to induce hypoglycaemia by the extract were established. Blood sugar estimation was done by ortho-toluidine method as follows[175].

Principle :-

Glucose reacts with aromatic amines such as o-toluidine in hot acetic acid solution to form a glycosylamine which undergoes condensation to form a green coloured product. The green colour is proportional to glucose concentration. Comparison with that of standard gives concentration of the unknown.

Reagents :-

i) **Trichloroacetic acid (TCA)** :- 3% in distilled water.

ii) **O-toluidine reagent** :- Pure thio urea (1.5 gms) was dissolved in about 200 ml glacial acetic acid with gentle warming. This was allowed to cool and transferred to a 1 litre volumetric flask. 60 ml of o-toluidine was added drop-wise with constant stirring to dissipate the heat. The volume was made to 1 litre with glacial acetic acid. This was stored in an amber coloured bottle. It has to be kept in dark for 48 hours, before use.

iii) **Stock standard** :- 100 mg% glucose in 0.25% benzoic acid made in distilled water.

iv) **Working standard** :- Stock standard was diluted 1:10 in distilled water to make 100 µg/ml.

Procedure :-

Blood (0.05 ml) was drawn from the orbito-sinus of the mouse, using a 50 µl graduated capillary. After blotting off excess blood, it

was added to 0.95 ml 3% TCA. After 10 minutes, it was centrifuged at 2000 rpm for 15 minutes and 0.5 ml of the protein free supernatent was added in the sample tube. Standard concentration (20 to 80 µg/ml) were added in 0.5 ml volume. 0.5 ml of D/W was added to the tube marked blank. Then 2.5 ml of o-toluidine reagent was added in all tubes. After plugging with non-absorbent cotton, the tubes were placed in a boiling waterbath for 10 minutes. After cooling, the absorbance was read at 630 nm.

5) Studies on enzyme activities :-

Preparation of liver homogenate :-

The levels of enzyme aspartate transaminase (aspT), alanine transaminase (alaT), serine dehydratase, phosphoenolpyruvate carboxykinase (PEPCK), *Phosphorylase a* and glycogen were studied in mice liver. At the end of definite time intervals after treatment, the 18 hours fasted mice of the different group were sacrificed. The liver were excised immediately and washed in ice-cold saline (0.154 M NaCl solution), blot dried and weighed and kept in a ice-cooled petriplate. It was then homogenized in 10 volumes (w/v) of respective buffer required for that particular assay. Homogenisation was done using potter-Elvehjem homogeniser at 4000 rpm with a stroke speed of 20 per minute. It was homogenised at 5°c, twice for 60 seconds with a gap of 15 seconds. The homogenate was centrifuged at 20,000 rpm for 20 minutes at 5°c and the clear supernatent

assayed for the following parameters colorimetrically or spectrophotometrically.

5.1) Aspartate transaminase and Alanine transaminase :-

The liver was homogenised in 0.25 M sucrose with 1 mM EDTA twice for 60 seconds, to give a 10% homogenate. To 1 ml of homogenate 50 µl 10% Triton X 100 was added[176] and the tubes were kept at $0^{o}c$ for 30 minutes. The homogenate was then centrifuged at 15000 rpm for 20 minutes and the supernatent was assayed for aspT and alaT.

5.1.a) Aspartate transaminase :-

It was estimated by the method of Rej, R. and Horder, M[177].

Principle :-

The enzyme aspT converts the L-aspartate in the presence of α-keto glutarate to L-glutamate and oxaloacetate. The auxillary enzyme malate dehydrogenase (MDH) converts oxaloacetate to malate by utilizing the NADH and converting it to NAD^{+}. The decrease in absorbance is read at 340 nm for 3 minutes.

Reagents :-

1) **0.25 M sucrose + 1 mM EDTA :-** The solution was stored at -20°c.

2) **10% Triton X 100**

3) **Tris Buffer 0.1 M, PH 7.8** :- The buffer was stored at 4°c.

4) **(297 mM) Aspartate in 0.1 M Tris Buffer** :- 3.95 gm L-Aspartic acid was dissolved in 0.1 M Tris base. The pH was adjusted to 7.8 with 10 M NaOH and volume was made to 100ml with distilled water. 10 ml aliquots were stored at -20°c.

5) **2-Oxoglutarate (144 mM) in 0.1 M Tris Buffer** :- 210 mg 2-Oxoglutarate was dissolved in 0.1 M Trisbuffer and the PH was adjusted to 7.8 with 2N NaOH and volume was made to 10 ml. 1 ml aliquots were made and stored at -20°c.

6) **Pyridoxal 5-phosphate (5.44 mM)** :- 7.2 mg pyridoxal 5-phosphate was dissolved in 5 ml 0.1 M Tris buffer (pH 7.8). 0.3 ml aliquots were made and stored at -20°c.

7) **MDH/LDH/glycerol solution** :- To 0.5 ml of 50% glycerol solution 10 µl Lactate dehydrogenase (LDH 10 mg/ml) and 10 µl MDH (10 mg/ml) from Bohreinger Mannheim was added. The vial was mixed and stored at 4°c.

Procedure :-

A complete reagent was made by mixing 10 ml of Aspartate/ tris solution, 0.25 ml of Pyridoxal 5-phosphate solution, 50 µl of the auxillary enzyme diluted in 50% glycerol solution. 1.5 mg of NADH was added to the above solution and the reagent was mixed. 1 ml of this reagent was used for each assay. 25 µl of supernatent obtained after centrifugation was diluted to 1 ml with 0.1 M tris buffer PH 7.8. Thus 0.25% homogenate was prepared. To the 1.5 ml capacity cuvette 1 ml of reagent, 100 µl of diluted supernatent was added and the mixture was incubated for 2 minutes. 100 µl of α keto glutarate was then added, the solution was mixed and then read immediately at 340 nm for 3 minutes at 1 minute interval. Air-blank was used.

To estimate the protein content of sample, protein in 0.1 ml of sample was precipitated with 1 ml of 10% TCA [10% tri-chloroacetic acid in distilled water], the supernatent was discarded, after centrifugation at 1500 rpm for 15 minutes. The protein precipitate was dissolved in 0.1 ml of IN NaOH and diluted to 5 ml with distilled water. A volume of 0.5 ml was taken for protein estimation by Folin-Lowry's method.

Calculation :-

1 unit of enzyme is defined as the amount of enzyme which converts 1 μmol of aspartate to oxaloacetate per minute in terms of the NADH oxidised[178].

$$\frac{1000 \times V}{\varepsilon \times d \times v} \times \Delta E/\Delta t = U/L$$

Where V = Assay volume

ε = 6.22 cm^2 / μ mole. extinction coef for NADH

d = light path

v = volume of sample used in assay

$\Delta E/\Delta t$ = Extinction change per minute

$$\frac{1000 \times 1.2}{6.22 \times 0.5 \times 0.1} \times \Delta E/\Delta t = U/L$$

$$\frac{1200}{0.311} \times \Delta E/\Delta t = U/L$$

3858.5 x $\Delta E/\Delta t$ = U/litre of supernatent

$$\frac{3858.5}{10} \times \Delta E/\Delta t$$ = units/100 ml of homogenate i.e. per 0.25 gms of tissue

$$3858.5 \times \Delta E/\Delta t \times \frac{4}{10}$$ = U/gm tissue

Specific activity = $\frac{\text{U/gm protein}}{\text{mg soluble protein/gm tissue}}$

5.1.b:- Alanine transaminase :-

It was estimated by the method of Horder, M and Rej, R[179].

Principle :-

The enzyme alanine transaminase converts the L-alanine in the presence of α keto glutarate to L-glutamate and pyruvate. The auxillary enzyme lactate dehydrogenase converts pyruvate to lactate by converting the NADH to NAD^+. The decrease in absorbance is read at 340 nm for 3 minutes.

Reagents :-

1) **0.25 M Sucrose, 1 mM EDTA** :- The solution was stored at -20°c.

2) **10% Triton X 100.**

3) **Tris buffer 0.11 M, PH 7.3** :- The buffer was stored at 4°c.

4) **(615 mM) L-alanine in 0.11 M Tris buffer** :- 5.48 gms L-alanine was dissolved in 0.11 M Tris base. The PH was adjusted to 7.3 with 1 N HCl. Volume was made to 100 ml with D/W and aliquots of 10 ml were stored at -20°c.

5) **2-Oxoglutarate (180 mM) in 0.11 M Tris buffer**, :- 263 mg 2-Oxo glutarate was dissolved in 0.11 M tris buffer and PH adjusted to 7.3 with 10 and 1 N NaOH. Volume was made to 10 ml with distilled water and 1 ml aliquotes were stored at -20°c.

6) **Pyridoxal 5-phosphate (5.0 m M)** :- 6.7 mg pyridoxal 5-phosphate was dissolved in 5.0 ml of 0.11 M Tris buffer PH 7.3. 0.3 ml aliquotes were stored at -20°c.

7) **LDH enzyme solution** :- To 0.2 ml of 50% glycerol solution, 0.25 ml of porcine LDH (10 mg/ml) (Boehringer Mannheim) was added and the mixture was stored at 4°c.

Procedure :-

A complete reagent was made by mixing 10 ml of alanine/ tris solution, 0.25 ml of pyridoxal 5-phosphate, 50 µl of the auxillary enzyme LDH diluted in 50% glycerol and 2 mg of NADH. 1 ml of this reagent was used for each assay. 50 µl of the supernatent obtained, after centrifugation was diluted to 0.5 ml with 0.11 M tris buffer PH 7.3. On further 1:2 dilution 0.5% homogenate was obtained. To the cuvette 1 ml of reagent and 100 µl of dilute supernatent was added and the mixture was incubated for 2 minutes in 1.5 ml capacity cuvette. 100 µl of α keto glutarate solution was then added, the solution mixed and then read immediately at 340 nm for 3 minutes at 1 minute interval. Air-blank was used.

Calculation :-

1 unit of enzyme is defined as the amount of enzyme which converts 1 µmol of alanine per minute. The calculation is same as the one given for the enzyme aspT except the last step.

$$\frac{3858.5}{10} \times \Delta E/\Delta t = \text{units/100 ml of homogenate}$$
0.5 gms of tissue

$$3858.5 \times \Delta E/\Delta t \times \frac{2}{10} = \text{U/gm tissue}$$

$$\text{Specific activity} = \frac{\text{U/gm protein}}{\text{mg soluble protein/gm tissue}}$$

5.2 Serine dehydratase :-

It is a gluconeogeneic enzyme. It converts the amino-acid serine to pyruvate. It was estimated by the method of Suda, M and Nakagawa, H[87].

Principle :-

The enzyme serine dehydratase from the homogenate converts the amino-acid L-serine to pyruvate in presence of pyridoxal 5 - phosphate. The pyruvate reacts with Dinitrophenyl hydrazine (DNPH) in presence of NaOH to form a reddish brown colouration of pyruvic dinitrophenyl hydrazone which is estimated at 520 nm.

Reagents :-

1) **KH_2PO_4, K_2HPO_4 buffer 0.05 M (PH 7.2)** :- 0.15 M KCl and 1 mM EDTA were also added. This buffer was used to homogenize the tissue. The buffer was stored at -20°c.

2) **KH_2PO_4, K_2HPO_4 buffer 0.2 M (PH 8.0)** :- 2 mM EDTA was added. The buffer was used in the assay. It was stored at -20°c.

3) **KH_2PO_4, K_2HPO_4 buffer 0.1 M (PH 8.0)** :- This buffer was used to dissolve L-serine and pyridoxal 5 - phosphate used in the assay system. It was stored at -20°c.

4) **2N HCl**

5) **2N NaOH**

6) **20% TCA**

7) **2, 4, DNPH** :- 0.033% DNPH solution was prepared in 2N HCl. The solution was warmed in a waterbath for the DNPH to dissolve. The solution was stored at 4°c.

8) **L-serine** :- 1 M L-serine was dissolved in 0.1 M phosphate buffer (PH 8.0). This solution was prepared fresh before each assay.

9) **Pyridoxal 5 - phosphate (0.5 mM)** :- 1.33 mg pyridoxal 5 - phosphate was dissolved in 0.1 M phosphate buffer (PH 8.0). 2 ml aliquotes were stored at -20°c.

10) **Pyruvate standard** :- 11 mg sodium pyruvate was dissolved in 0.2 M phosphate buffer (PH 8.0) containing 1 mM EDTA and volume was made to 100 ml with the same. This solution corresponds to 1 µmol/ml.

Procedure :-

The weighed liver tissue was homogenized in 10 volumes of 0.05 M phosphate buffer PH 7.2 containing 1 mM EDTA and

0.15 M KCl for 120 seconds. The homogenate was centrifuged at 20,000 r.p.m at 5°c for 20 minutes in centrikon - T 324 centrifuge. The clear supernatent was used for assay. A control was run with each test. The standard graph was plotted from the range of 0.025 µmoles to 0.2 µmoles of pyruvate per assay.

The protocol for test was as follows :-

	Test	Control
0.5 mM pyridoxal 5-phosphate	0.1 ml.	0.1 ml
0.2 M phosphate buffer (PH 8.0)	0.75 ml	0.75 ml
10% Homogenate	0.050 ml	0.050 ml
Total volume	0.9 ml	0.9 ml
Incubate	37°c/5 minutes	
20% TCA	—	0.5 ml
1 M Serine (PH 8.0)	0.1 ml	0.1 ml
Incubate	37°c/5 minutes	
20% TCA	0.5 ml	—
	cool in ice for 10 minutes.	
	centrifuge 2000 r.p.m./10 minutes	

In a new set of test-tubes :-

	Test	Control	Blank	Standard
0.2 M Phosphate buffer (PH 8.0) :		—	0·5ml	0.4 ml
Pyruvate Standard (1 µmol/ml) :		—	—	-- 0.1 ml
Test supernatent :	0.5 ml	0.5 ml	—	—
DNPH (0.033%) :	0.5 ml	0.5 ml	0.5 ml	0.5 ml
	Incubate at Room Temperature (R.T.) for 5 minutes			
2N NaOH	2 ml	2 ml	2 ml	2ml

The tubes were read at 520 nm

Calculations :-

1 unit of enzyme is defined as the amount of enzyme which converts 1 µmol of serine to pyruvate per minute.

$\frac{\text{T-C Rdg}}{\text{Std-BL}} \times \text{conc of std} = \text{Test pyruvate concentration}$

$x = \frac{\text{corrected O.D of test}}{\text{corrected O.D. of Std}} \times 0.1 = \text{µmoles pyruvate/assay or per 0.5 ml of supernatent}$

x = µmoles of pyruvate formed by 16.5 µl of homogenate

$y = \frac{x}{5}$ = µmoles pyruvate formed/minute/1.65 mg tissue

$\frac{Y \times 1000}{1.65}$ = units/gm tissue

$\text{Specific activity U/mg protein} = \frac{\text{units/gm tissue}}{\text{mg soluble protein per gm tissue}}$

5.3 Phosphoenolpyruvate carboxykinase (PEP-CK) :-

It is the rate limiting enzyme of gluconeogenesis. It converts the metabolite oxaloacetate to phosphoenol pyruvate. It was estimated by the method of Moore, et. al[180].

Principle :-

The enzyme PEP-CK catalyses the ITP - dependent decarboxylation of oxaloacetate to phosphoenol pyruvate.

In vitro the enzyme can also be estimated in the reverse direction. The enzyme PEP-CK converts phosphoenol pyruvate to oxaloacetate in the presence of IDP. The oxaloacetate thus formed is converted to L-malate by the auxillary enzyme MDH in the presence of NADH. The decrease in absorbance is read at 340 nm.

Reagents :-

1) **Triethanolamine buffer 0.01 M (PH 7.5)** : 0.25 M sucrose, 1 mM EDTA, 1mM dithiothreitol (DTT) were also added to the buffer. This buffer was used to homogenize the tissue. The buffer was stored at -20°c.

2) **N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HePeS) buffer 0.25 M (PH 7.2)** : The buffer was stored as 2 ml aliquotes at -20°c.

3) **$MnCl_2$. $4H_20$ (20 mM)** :- The solution was aliquoted in 1 ml quantity and was stored at -20°c.

4) **DTT (10mM)** :- 15.42 mg were dissolved in 10 ml distilled-water and the vials of 1 ml quantity were stored at -20°c.

5) **$NaHCO_3$ (1 M)** :- This solution was prepared fresh before use.

Procedure :-

The weighed liver tissue was homogenized in 5 volumes of 0.01 M triethanol amine buffer PH 7.5 containing 0.25 M sucrose, 1 mM EDTA and 1 mM DTT for 60 seconds at 4°c. The homogenate was centrifuged at 18000 r.p.m. at 5°c for 30 minutes. The clear supernatent was used for assay. A complete reagent for 10 tests was made by mixing 2 ml of Hepes buffer 0.25 M (PH 7.5), 1 ml of 10 mM DTT, 1 ml of 20 mM $MnCl_2.4H_20$. 2 mg NADH, 5 mg IDP and 5.3 mg phosphoenolpyruvate (PEP) were dissolved in 6 ml distilled water. It was then added to the above 4 ml mixture and the solution was mixed. 75 µl of MDH (10 mg/ml) in 50% glycerol solution was added to the above reagent and the solution was mixed. 1 ml of this solution was used for each assay. To the cuvette 1 ml of reagent, 25 µl of supernatent were added and mixed. The mixture was incubated for 2 minutes. 50 µl of 1M $NaHCO_3$ was then added, the

solution mixed and then read immediately at 340 nm for 3 minutes at 1 minute interval. Air blank was used.

Calculation :-

1 unit of enzyme is defined as the amount of enzyme which converts 1 µmol of PEP to oxaloacetate per minute at 25°c.

$$\frac{1000 \times V}{e \times d \times v} \times \Delta E/\Delta t = U/L$$

$$\frac{1000 \times 1.075}{6.22 \times 1 \times 0.025} \times \Delta E/\Delta t = U/L$$

$$6913 \times \Delta E/\Delta t = U/1000 \text{ ml of supernatent}$$

$$\frac{6913 \times \Delta E/\Delta t}{10} = U/100 \text{ ml of supernatent or U per 20 gms of tissue}$$

$$\frac{6913 \times \Delta E/\Delta t}{10 \times 20} = U/\text{gm tissue}$$

$$\text{Specific activity U/mg protein} = \frac{U/\text{gm tissue}}{\text{mg soluble protein/gm tissue}}$$

5.4 *Phosphorylase a* :-

This enzyme catalyses the breakdown of glycogen to glucose 1-phosphate from which glucose 6-phosphate and then free blood glucose are formed. It was estimated by the method described by Bergmeyer[181].

Principle :-

The enzyme *phosphorylase a* from the homogenate breaks down glycogen to yield glucose 1-phosphate. The auxillary enzyme phosphoglucomutase in the reaction-mixture converts glucose 1-phosphate to glucose 6-phosphate. It is converted by the second auxillary enzyme glucose 6-phosphate dehydrogenase (G6PDH) and $NADP^+$ to give 6-phosphogluconate and NADPH. The increase in absorbance is read at 365 nm.

Reagents :-

1) **NaF (0.1M)** :- The solution was used to homogenize the tissue. It was stored at 4°c.

2) **KH_2PO_4, K_2HPO_4 buffer 0.05 M (PH 6.8)** :- 7.5 ml aliquotes of buffer were prepared and stored at -20°c.

3) **E.D.T.A. (10 mM)** :- The solution was prepared in 0.05 M phosphate buffer PH 6.8 and 100 µl aliquotes were stored at -20°c.

4) **Glycogen** :- 18 mg of glycogen was dissolved in 3 ml D/W and 300 µl aliquctes were stored at -20°c.

5) **$MgCl_2$, $4H_2O$ (1 M)** :- The solution was prepared and 150 µl aliquotes were stored at -20°c.

6) **Glucose 1,6-biphosphate (1.357 mM)** :- 1 mg/ml solution was prepared in D/W and 25 µl aliquotes were stored at -20°c.

7) **Phosphoglucomutase** :- The enzyme was obtained in a 2.5 M $(NH_4)_2SO_4$ solution PH 6.0 containing 0.01% EDTA from sigma. The enzyme was made SO_4 free before use. 64 µl or 100 units of the enzyme was diluted to 0.2 ml with D/W and dialysed against 50 mM acetate buffer pH 5.3 at 4°c, till traces of SO_4 disappeared. The solution was then diluted to 0.5 ml with acetate buffer (50 mM) pH 5.3 and 50 µl aliquotes were stored at -20°c.

8) **Glucose 6-phosphate dehydrogenase** :- The enzyme was obtained in a lyophilised form from sigma. The 2000 units were diluted to 1 ml and 25 µl aliquotes were stored at -20°c.

Procedure :-

The weighed liver tissue was homogenised in 5 volumes of 0.1 M NaF for 120 seconds. The homogenate was centrifuged at 8000 X g for 10 minutes and the supernatent was used for assay. A complete reagent was prepared by mixing 7.5 ml of phosphate buffer 0.05 M (pH 6.8) with 75 µl EDTA, 0.25 ml glycogen, 0.125 ml $MgCl_2$, 25 µl Glucose-1, 6-biphosphate, 50 µl phosphoglucomutase, 25 µl G6PD and 2.5 mg $NADP^+$ was added and the reagent was mixed

thoroughly. 0.84 ml of this reagent and 15 µl of supernatent were added to 1.0 ml cuvette, the solution was mixed and then read immediately at 365 nm for 5 minutes at 1 minute interval. D/W blank was used.

Calculation :-

1 unit of enzyme is defined as the amount of enzyme which causes liberation of 1 µmol of glucose 1-phosphate per minute.

$$\frac{1000 \times V}{e \times d \times v} \times \Delta E/\Delta t = U/L$$

e = 3.45 cm^2/µmole. extinction coef for NADPH

$$\frac{1000 \times 0.855}{3.45 \times 1 \times 0.015} \times \Delta E/\Delta t = U/L$$

$$16521 \times \Delta E/\Delta t = U/L$$

$$\frac{16521 \times \Delta E/\Delta t}{10} = U/100 \text{ ml of supernatent or U per 20 gms of tissue}$$

$$\frac{16521 \times \Delta E/\Delta t}{10 \times 20} = U/gm \text{ tissue}$$

$$\text{Specific Activity} = \frac{U/gm \text{ tissue}}{mg \text{ soluble protein per gm tissue}}$$

5.5 Glycogen :-

Glycogen is the storage polysaccharide found in the liver. It was estimated by the combined method of Seifter[182] and Trevelyan W.E. et.al[183].

Principle :-

The liver tissue is digested with 30% KOH and the glycogen is precipitated in the presence of Na_2SO_4 and 95% ethanol. The reprecipitated glycogen reacted with anthrone in the presence of sulfuric acid to give a bluish green colour which was estimated at 620 nm.

Reagents :-

1) **KOH 30%**

2) **Na_2SO_4 (saturated)**

3) **Ethanol 95%**

4) **Anthrone Reagent** :- 20 ml D/W was cooled over ice, 50 ml of concentrated H_2SO_4 was added to it, the solution was swirled and cooled, then 20 mg Anthrone was added and the solution mixed. The reagent was prepared fresh before use.

5) **Glycogen Standard** :- 10 mg% glycogen made in D/W.

Procedure :-

Liver tissue was washed with ice-cold saline and blot dried. 100-150 mg of weighed tissue was added to 0.6 ml of 30% KOH present in a 15 ml graduated centrifuge tube. The solution and the tissue were then boiled in a boiling water bath for 20 minutes. To the cooled tube 25 µl of saturated Na_2SO_4 was added and the solution was mixed on vortex. 1.25 ml of 95% ethanol was then added and

the tubes were mixed gently to precipitate the glycogen. The solution was warmed by keeping it in the boiling water bath. It was then cooled and centrifuged at 2000 r.p.m for 15 minutes. The supernatent was discarded. The precipitate of glycogen was dissolved in 1 ml D/W and reprecipitated with 95% ethanol. The solution was mixed and cooled and centrifuged at 2000 r.p.m. for 10 minutes. The supernatent was discarded and the tubes were kept inverted on filter paper for few minutes. Anthrone reagent was prepared and 5 ml of it was added to each tube. The glycogen from the test-tubes were dissolved in 5 ml of D/W. 1 ml of test solution was layered over the cooled reagent. Blank consisted of 1 ml D/W and 5 ml of the reagent. For the standard graph 0.025 to 0.1 mg/ml solutions each were layered over the cooled reagent. The tubes were swirled gently to mix, plugged and boiled in a boiling water bath for 10 minutes and cooled. They were read at 620 nm.

Calculations :-

$$\frac{\text{Abs T}}{\text{Abs S}} \times \text{conc of standard}$$

$$\frac{T}{S} \times 0.050 = X \text{ mg per ml of test solution}$$

$5 \times X = 5X$ mg per 5 ml of test solution or per y mg of liver tissue

$$\frac{5X \times 1000}{Y} = z \text{ mg glycogen per 1000 mg liver tissue or mg glycogen per gm tissue}$$

6 Estimation of serum insulin levels :-

Mice from various groups were bled and the blood was allowed to clot. The serum was collected and stored at -20°c until assay. Serum insulin levels were assayed by radio immunoassay (RIA) by the double antibody technique using the kit supplied by Board of Radiation and Isotope Technology, BARC, Bombay, India.

Principle :-

RIA is based upon the competition of unlabelled insulin in the standard or samples and radio iodinated (I-125) insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound insulin is separated by the second antibody from the free insulin. Polytethylene glycol (PEG) precipitates this complex, which can be read in a gamma counter.

Reagents :-

Insulin assay kit : this provides

1) **Porcine Insulin standard 200 μ U/ml**

2) **Antiporcine Insulin serum (guinea pig)**

3) **Insulin free serum**

4) **I-125 radio-iodinated insulin**

5) **Second antibody - antiguinea pig gamma globulin (rabbit)**

6) **Assay buffer**

7) **Polyethylene glycol (PEG) solution**

8) **Control serum (2 nos.)**

Procedure :-

First the serum samples were collected and stored at -20°c. For this, mice from various groups were bled from orbital sinus. The blood was allowed to clot at room-temperature. After 2 to 3 hours the blood was centrifuged at 2000 r.p.m for 15 minutes at 4°c and the serum was then collected with a auto-pipette. It was recentrifuged if necessary. The serum was stored as 200 µl aliquots at -20°c. The lyophilized reagents were reconstituted as specified. Insulin standards of concentration 100.00, 50.0, 25.0, 12.5 and 7.5 µ U/ml were prepared from the given 200.00 µ U/ml standard.

For RIA, blank and standards were set up in duplicates. To one tube only I-125 insulin was added in 0.1 ml amount. It gave the total count of labelled insulin. To other tube assay buffer 0.4 ml, insulin free-serum in 0.1 ml amount and I-125 insulin in 0.1 ml amount were added along with II antibody (0.1 ml) and PEG (1.0 ml). This reading gave the non-specific binding (NSB) or the blank count. To next tube was added assay buffer (0.3 ml) insulin free serum (0.1 ml), anti-insulin serum (0.1 ml) and I-125 insulin (0.1 ml) along with II antibody (0.1 ml) and PEG (1.0 ml). This tube gave the total binding capacity of the antibody

to the labelled insulin or the total binding count. The standard consisted of 6 tubes to each 0.1 ml of appropriately dilute standard was added, 0.2 ml of assay buffer, 0.1 ml of insulin free serum and 0.1 ml of anti-insulin serum was added and the tubes were mixed gently and incubated overnight at 4°c. Next day 0.1 ml of I-125 labelled insulin was added to all tubes. The tubes were mixed and kept at R.T for 3 hours. Then 0.1 ml of II antibody was added to all tubes, mixed and 1.0 ml of PEG was added. The tubes were mixed and kept at R.T for 20 minutes. They were then centrifuged at 1500 x g for 20 minutes. The supernatent was discarded in a container. The solution was blotted from the outer rim of the tubes and the precipitate in the pellet was read in a gamma counter. The same procedure was used for control serum and test-serum samples except that insulin free serum was not added and instead 0.1 ml extra assay buffer was added to each tube.

Calculation :-

The Blank count was substracted from all the counts to get the corrected count. It is known as the Binding count (B) The count obtained by adding only the labelled I-125 insulin and the antibody constituted the total Binding count or B_0

$$\% \; B/B_0 = \frac{\text{Corrected count of std or sample}}{\text{Corrected count of total binding of I-125 insulin } (B_0)} \times 100$$

The graph is plotted with % B/B_0 on Y axis and μ units/ml of insulin on X axis using a logit log paper. The % B/B_0 values of test-samples are used to find out the insulin values from the graph.

Results were expressed as Mean ± S.E.M and significance was calculated using student's unpaired 't' test[184].

Results

The *B-pertussis* extract obtained after purification was found to be a protein with minute amounts of carbohydrates (1.5 mg%) and lipids (400 µg%). There were also no toxic symptoms or death of the animals even upto a period of 4 weeks with the dose level studied. The *B.P.* ext. was administered in such a way that its maximum action (6 hours) coincided with the peak action of epinephrine, for the different parameters studied.

Blood Sugar Studies :- Dose response studies were carried out initially to assess the hypoglycaemic effect of the extract in mice. At doses of 0.5 mg/kg b.wt and 1.0 mg/kg b.wt, on the basis of protein concentration, the blood sugar levels at the end of 6 hours were lowered to 63.25 ± 1.92 and 55.92 ± 1.72 mg% (± S.E.M, n=5) respectively as compared to control values of 103.02 ± 3.06 mg%. With 1.5 mg/kg b.wt, the blood sugar values were further lowered to 49.55 ± 2.31 mg% at the end of 6 hours. As shown in Table 1 and Fig. 7, the blood sugar levels observed with the dose of 1.0 mg/kg b.wt at the end of 6 and 12 hours were 55.92 ± 1.72 mg% and 51.12 ± 2.16 mg% respectively. It corresponded to 45.72% and 50.38% lowering as compared to the control values and were highly significant ($p<0.001$). As there was not much of a difference in the 6 hours blood sugar values between the 1.0 mg/kg b.wt and 1.5 mg/kg b.wt dose, it was decided to use 1.0 mg/kg b.wt dose in all further studies.

TABLE - I

EFFECT OF *B.PERTUSSIS* EXTRACT ON BLOOD SUGAR LEVELS IN MICE

Dose :- B.pertussis ext = 1.0 mg/Kg b. wt (i.v.)

	BLOOD SUGAR IN mg %				
GROUP TREATMENT	0 Hrs	6 Hrs	12 Hrs	18 Hrs	24 Hrs
B. PERTUSSIS EXT (1.0mg/kg b.wt) (i.v.)	103.02 ± 3.06	*55.92 ± 1.72 45.72% LOWERING	##51.12 ± 2.16 50.38% LOWERING	93.48 ± 2.79	107.22 ± 4.89

Results expressed as mean ± S.E.M, n = 5

In comparison with control group * & ## $p < 0.001$

(Student's unpaired 't' test)

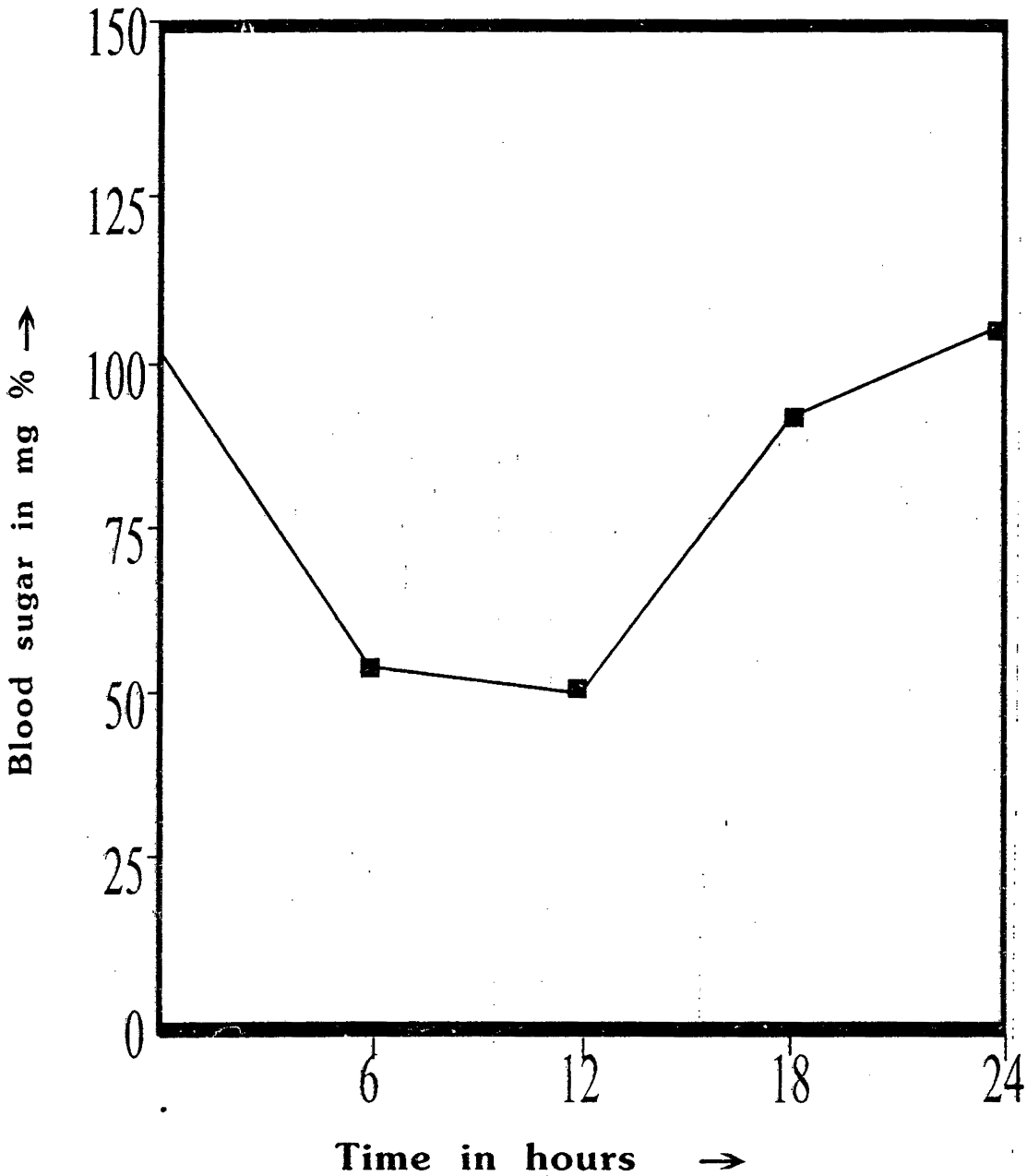


Fig. 7 : Effect of *B. pertussis extract* (200 µg/ml) on blood sugar level at different time interval.

The hyperglycaemic effect of epinephrine administered intraperitoneally was initially examined with different doses at the end of 30 minutes. Three doses of epinephrine 0.3 mg/kg b.wt, 0.4 mg/kg b.wt and 0.65 mg/kg b.wt were administered in 18 hours fasted mice. This caused hyperglycaemia at the end of 30 minutes and the blood sugar increased to 146.22 ± 3.78 mg%, 179.70 ± 4.87 mg% and 198.52 ± 3.2 mg% respectively (± S.E.M, n=5) as compared to the saline values of 98.54 ± 4.14 mg% (± S.E.M, n=5). Though the hyperglycaemia was maximum with 0.65 mg/kg b.wt, 0.4 mg/kg b.wt dose was chosen for further studies. This was done because with 0.65 mg/kg b.wt, the mice appeared disturbed and had difficulty in movements.

Table II and Fig. 8 show the reversal of epinephrine-induced hyperglycaemia in mice. In overnight fasted mice, epinephrine was administered at the dose of 0.4 mg/kg b.wt (i.p.). It caused a maximum hyperglycaemia of 181.41 ± 4.98 mg% (± S.E.M, n=10) at the end of 30 minutes. The blood sugar returned to normal at the end of 90 minutes (109.66 ± 2.27 mg%). The control and *B-pertussis* extract treated values were 89.88 ± 2.78 and 53.33 ± 1.66 mg% respectively. Pretreatment with *B.P. ext* significantly lowered the blood sugar levels to 116.81 ± 3.13 mg% ($p<0.001$). The *B.P. ext.* thus caused 35.61% lowering of blood sugar in the pretreated group.

TABLE - II

REVERSAL OF EPINEPHRINE INDUCED HYPERGLYCAEMIA IN MICE BY *B. PERTUSSIS* EXTRACT

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	BLOOD SUGAR IN mg %		
GROUP TREATMENT	30 MINUTES	60 MINUTES	90 MINUTES
SALINE	89.88 ± 2.78	93.68 ± 2.55	94.04 ± 3.63
B.PERTUSSIS EXT.	53.33 ± 1.66	55.64 ± 1.58	58.19 ± 1.17
EPINEPHRINE	181.41 ± 4.98	136.22 ± 2.02	109.66 ± 2.27
B.P EXT+EPINEPHRINE	*116.81 ± 3.13	95.04 ± 3.23	96.17 ± 3.06

Results expressed as Mean ± S E.M., n = 10

In comparison with Epinephrine* group $p < 0.001$ (student's unpaired 't' test)

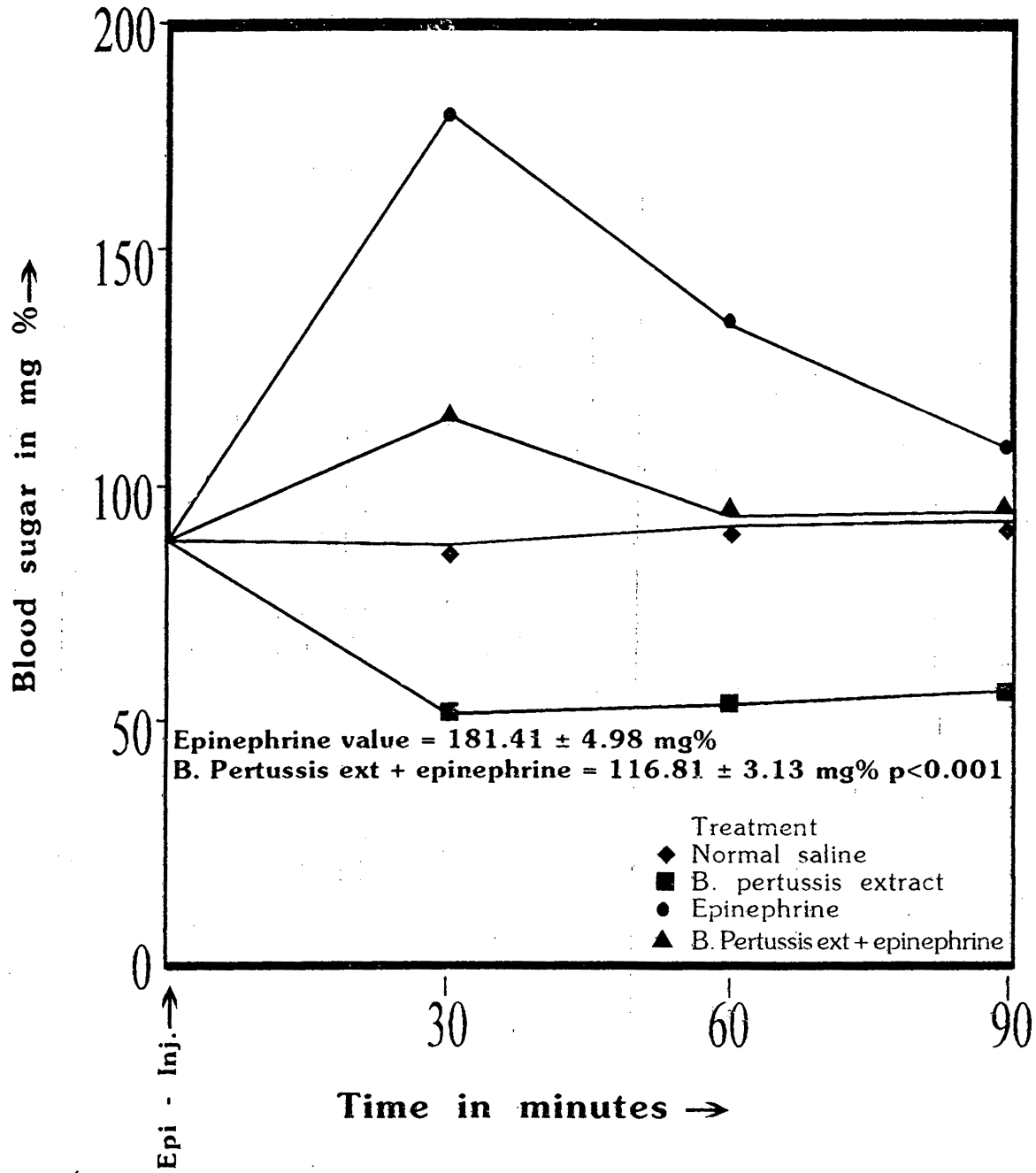


Fig. 8 : Reversal of Epinephrine-induced hyperglycaemia in mice by *B. pertussis extract*

Table III and Fig. 9 show the level of liver enzyme aspartate transaminase in various treated groups. The values in *B.P ext* treated group were lowered from 1.037 ± 0.029 U/mg protein to 0.81 ± 0.017 U/mg protein which was a 21.9% lowering. In normal mice epinephrine caused initial decrease in the enzyme level at the end of 1 hour followed by a maximum increase in the enzyme level at the end of 2 hours (1.24 ± 0.007 u/mg protein) (± S.E.M, n=7). Pretreatment with *B.P. ext.* significantly lowered the enzyme to 1.03 ± 0.020 U/mg protein ($p<0.001$) which was 16.94% lowering compared to the epinephrine group.

In severe alloxan diabetic mice, the enzyme increased to 1.43 ± 0.019 U/mg protein at the end of 48 hours. Treatment with *B.P. ext.* 6 hours before the estimation, significantly lowered the enzyme to 1.32 ± 0.014 U/mg protein ($p<0.001$).

Table IV shows the value of the enzyme alanine transaminase in the different treated groups. Control and *B.P. ext.* treated values were 0.38 ± 0.009 and 0.33 ± 0.007 U/mg protein. The enzyme did not increase upto 3 hours in epinephrine treated mice. Pretreatment with *B.P. ext.* did not significantly lower the enzyme (0.36 ± 0.012 U/mg protein) (± S.E.M, n=7).

The enzyme was not found to increase in severely alloxan diabetic mice (0.33 ± 0.016 U/mg protein) at the end of 48 hours.

TABLE - III

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER ASPARTATE TRANSAMINASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U/mg PROTEIN		
GROUP	TIME AFTER EPINEPHRINE ADMINISTRATION		
TREATMENT	1 HOUR	2 HOURS	3 HOURS
SALINE	----	1.037 ± 0.029	----
B.PERTUSSIS EXT.	0.87 ± 0.007	0.81 ± 0.017	0.89 ± 0.022
EPINEPHRINE	0.53 ± 0.017	1.24 ± 0.007	0.88 ± 0.012
B.P EXT+EPINEPHRINE	1.09 ± 0.011	*1.03 ± 0.020	1.06 ± 0.021
ALLOXAN (48 HRS)	1.43 ± 0.019		
ALLOXAN+*B.P EXT.*	**1.32 ± 0.014		

Results expressed as Mean ± S.E.M., n = 7

In comparison with Epinephrine* group $p < 0.001$

In comparison with Alloxan (48 hrs)** group $p < 0.001$ (student's unpaired 't' test)

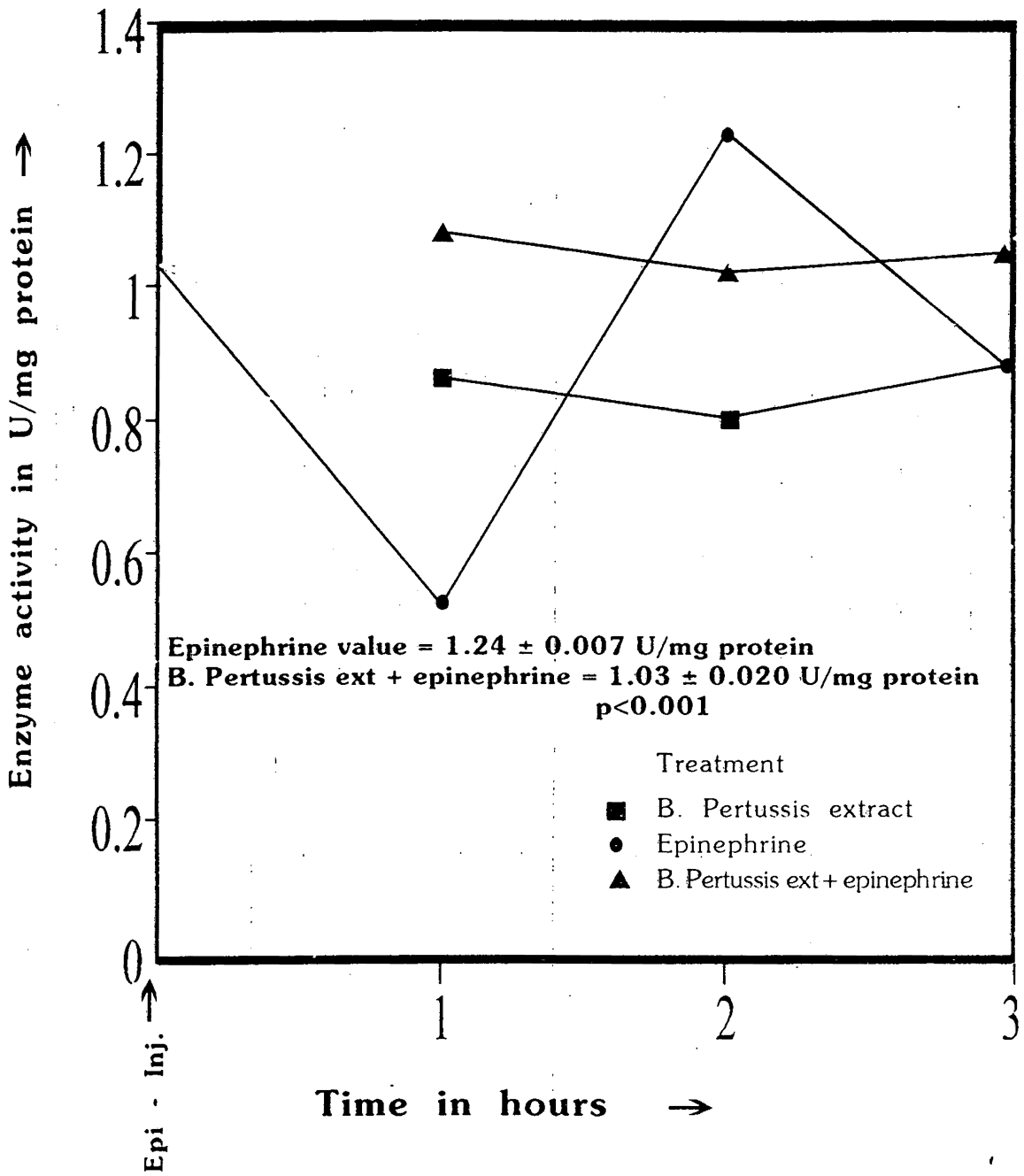


Fig. 9 : Effect of *B. pertussis extract* on liver enzyme *Aspartate Transaminase* after administration of epinephrine

TABLE - IV

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER ALANINE TRANSAMINASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) *B.pertussis ext.* = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U/mg PROTEIN		
GROUP	TIME AFTER EPNEPHRINE ADMINISTRATION		
TREATMENT	1 HOUR	2 HOURS	3 HOURS
SALINE	----	0.38 ± 0.009	----
B.PERTUSSIS EXT.	0.34 ± 0.011	0.33 ± 0.007	0.37 ± 0.016
EPINEPHRINE	0.28 ± 0.008	0.33 ± 0.015	0.28 ± 0.012
B.P EXT+EPINEPHRINE	0.38 ± 0.004	0.36 ± 0.012	0.37 ± 0.008
ALLOXAN (48 HRS)	0.33 ± 0.016		
ALLOXAN+*B.P EXT.*	0.33 ± 0.010		

Results expressed as Mean ± S.E.M., n = 7

Not significant

Treatment with *B.P. ext.* 6 hours before the estimation of enzyme could not lower the enzyme levels.

Table V and Fig. 10 show the effect of *B. Pertussis* extract on the levels of the liver gluconeogenic enzyme, serine dehydratase. In the *B.P. ext.* treated group, values were lowered to 0.112 ± 0.006 U/mg protein as compared to the saline values of 0.164 ± 0.005 U/mg protein, which was a 31.74% lowering. Administration of epinephrine at 0.4 mg/kg.b.wt (i.p) caused the enzyme serine dehydratase to increase to 0.24 + 0.005 U/mg protein (± S.E.M, n=7) at the end of 60 minutes. In the *B.P. ext.* pretreated group, the enzyme was lowered to 0.144 ± 0.005 U/mg protein which was a highly significant lowering of 40%.

In severe alloxan diabetic mice, the enzyme level increased to 0.23 ± 0.009 U/mg protein at the end of 48 hours, which was lowered significantly by administration of *B.P ext.* 6 hours before the estimation of the enzyme to 0.16 ± 0.011 U/mg protein ($p<0.001$) which was 30.43% lowering.

Table VI and fig. 11 gives the effect of *B.P. ext* on liver PEP-CK levels. In the *B.P ext.* treated group values were lowered from 24.1 ± 1.2 U x 10^{-3} mg protein to 18.7 ± 0.7 U x 10^{-3}/mg protein (± S.E.M, n=7) which was a 22% lowering. Administration of epinephrine cause the enzyme PEP carboxy kinase to increase to 36.3 ± 1.3 U x 10^{-3}/mg protein at the end of 2 hours. Pretreatment

TABLE - V

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER GLUCONEOGENEIC ENZYME SERINE DEHYDRATASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U/mg PROTEIN		
GROUP TREATMENT	TIME AFTER EPINEPHRINE ADMINISTRATION		
	30 MINUTES	60 MINUTES	90 MINUTES
SALINE	----	0.164 ± 0.005	----
B.PERTUSSIS EXT.	0.108 ± 0.003	0.112 ± 0.006	0.114 ± 0.007
EPINEPHRINE	0.176 ± 0.003	0.24 ± 0.005	0.14 ± 0.006
B.P EXT+EPINEPHRINE	0.153 ± 0.004	*0.144 ± 0.005	0.124 ± 0.008
ALLOXAN (48 HRS)	0.23 ± 0.009		
ALLOXAN+*B.P EXT.*	**0.16 ± 0.011		

Results expressed as Mean ± S.E.M., n = 7

In comparison with Epinephrine* group $p < 0.001$

In comparison with Alloxan (48 hrs)** group $p < 0.001$ (student's unpaired 't' test)

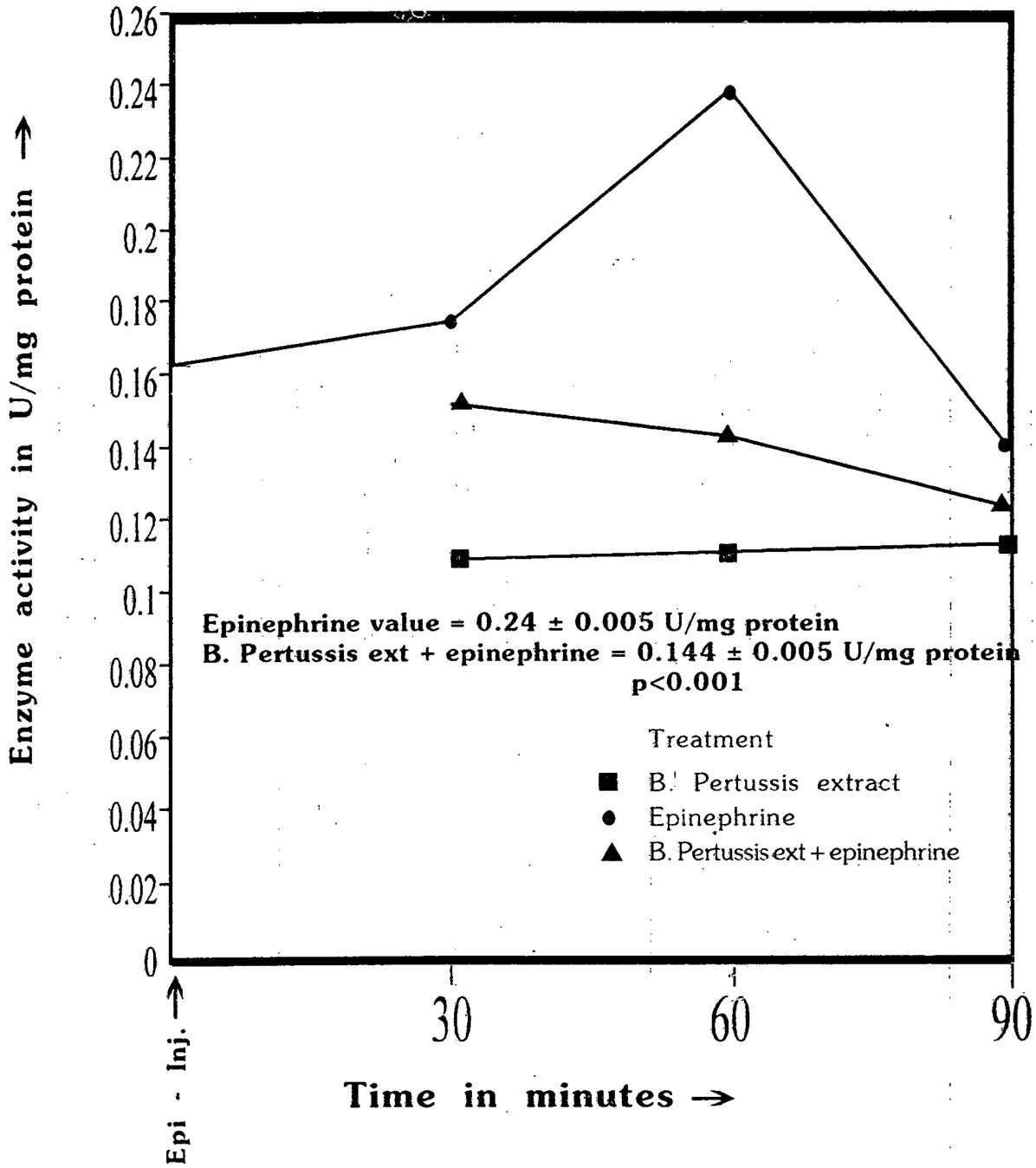


Fig. 10 : Effect of *B. pertussis extract* on liver enzyme *Serine dehydratase* after administration of epinephrine

TABLE - VI

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U x 10^{-3}/mg PROTEIN		
GROUP	TIME AFTER EPINEPHRINE ADMINISTRATION		
TREATMENT	1 HOUR	2 HOURS	3 HOURS
SALINE	----	24.1 ± 1.2	----
B.PERTUSSIS EXT.	---	18.7 ± 0.7	---
EPINEPHRINE	24 ± 1	36.3 ± 1.3	29.8 ± 0.9
B.P EXT+EPINEPHRINE	20.8 ± 1.1	*22.4 ± 0.8	18.6 ± 0.9
ALLOXAN (48 HRS)	45 ± 1.5		
ALLOXAN+*B.P EXT.*	**35.7 ± 1.16		

Results expressed as Mean ± S.E.M., n = 7

In comparison with Epinephrine* group $p < 0.001$

In comparison with Alloxan (48 hrs)** group $p < 0.001$ (student's unpaired 't' test)

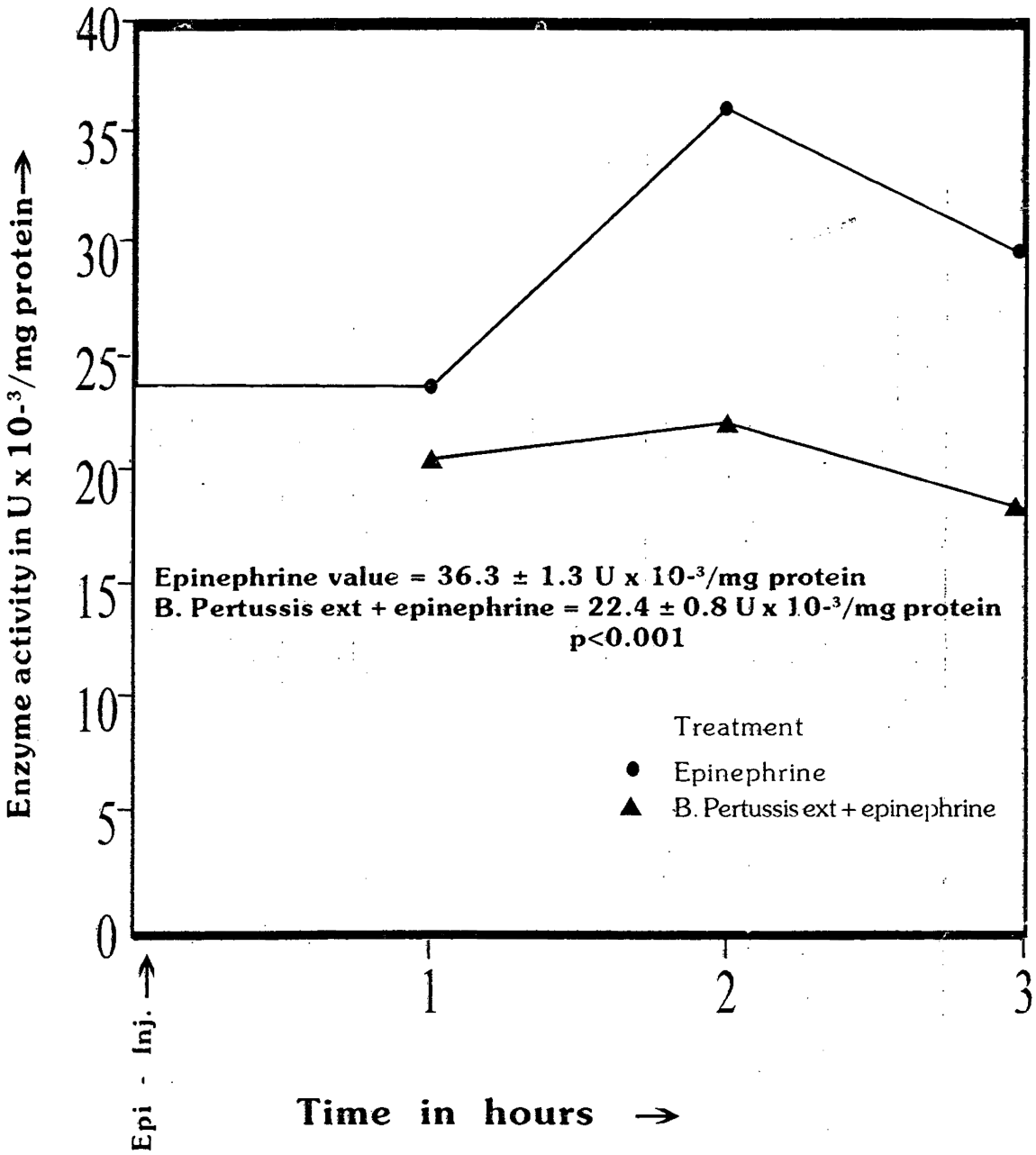


Fig. 11 : Effect of *B. pertussis extract* on liver enzyme PEP-CK after administration of epinephrine

with *B.P ext.* 6 hours before epinephrine lowered the enzyme levels to 22.4 ± 0.8 U x 10^{-3}/mg protein (38.3% lowering) which was significant.

In severe alloxan diabetic mice, the enzyme levels increased to 45 ± 1.5 U x 10^{-3}/mg protein at the end of 48 hours. It was significantly lowered by administration of *B.P. ext.* 6 hours before the estimation of enzyme to 35.7 ± 1.16 U x 10^{-3}/mg protein (21.4% lowering).

Table VII and Fig. 12 shows the effect of *B.P. ext.* on the levels of liver enzyme *phosphorylase a*. The enzyme levels in B.P. ext. treated group and control group were 35.1 ± 0.5 U x 10^{-3}/mg protein and 34 ± 0.9 U x 10^{-3}/mg protein. Administration of epinephrine caused the enzyme *phosphorylase a* to increase to 44.1 ± 1.0 U x 10^{-3}/mg protein, (± S.E.M, n = 7) at the end of 12.5 minutes. Pretreatment with *B.P. ext.* significantly lowered the enzyme to 33.8 ± 0.7 U x 10^{-3}/mg protein which was a 23.36% lowering.

Table VIII shows the effect of *B.P. ext.* on serum insulin and liver glycogen. Fig. 13 shows the insulin standard curve for concentrations ranging from 0-200 μ Units/ml. Based on this, the concentrations of insulin in test serum samples were calculated. Administration of *B.P. ext.* showed significant increase in the serum insulin levels of 26.25 ± 3.4 m units/ml at the end of 6 hours as compared to the

TABLE - VII

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER ENZYME *PHOSPHORYLASE a* AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U X 10^{-3} / mg PROTEIN		
GROUP	TIME AFTER EPINEPHRINE ADMINISTRATION		
TREATMENT	5 MINUTES	12.5 MINUTES	15 MINUTES
SALINE	---	**34 ± 0.9	---
B.PERTUSSIS EXT.	---	35.1 ± 0.5	---
EPINEPHRINE	35.5 ± 0.7	44.1 ± 1	24.1 ± 0.9
B.P EXT+EPINEPHRINE	34.3 ± 1	*33.8 ± 0.7	35.1 ± 0.9

In comparison with Epinephrine* group $p < 0.001$

In comparison with Epinephrine** group $p < 0.001$

Results expressed as Mean ± S.E.M, n = 7 (student's unpaired 't' test)

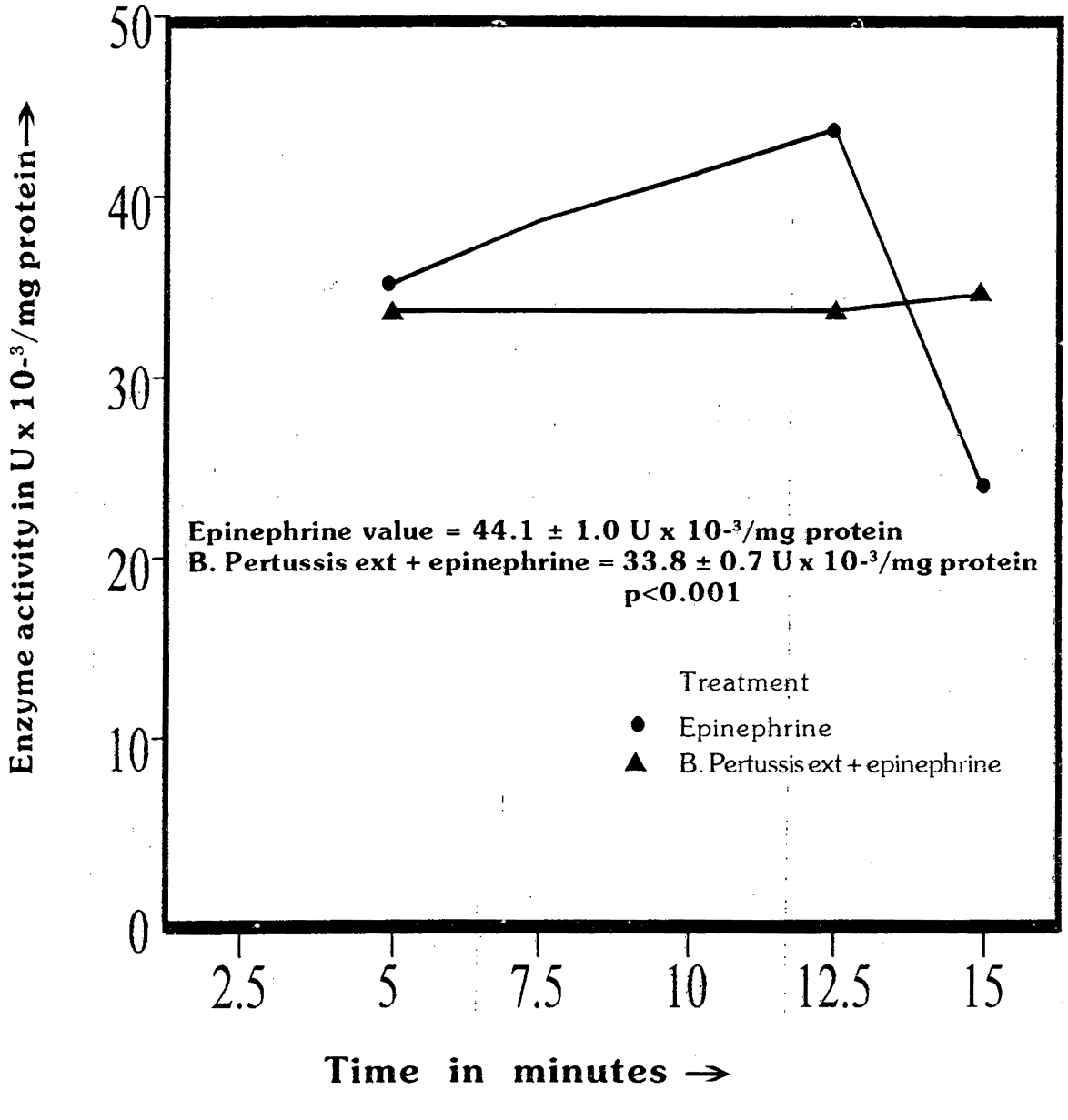


Fig. 12 : Effect of *B. pertussis extract* on liver enzyme *Phosphorylase a* after administration of epinephrine

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TABLE - VIII

EFFECT OF *B.PERTUSSIS* EXTRACT ON SERUM INSULIN AND LIVER GLYCOGEN AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	NORMAL SALINE GROUP	*B.PERTUSSIS* EXTRACT (6 HRS) GROUP	EPINEPHRINE (30 MINUTES)	*B.PERTUSSIS EXT.* + EPINEPHRINE (30 MINUTES)	
INSULIN μ units/ml serum	16.56 ± 1.41	**26.25 ± 3.40	21.61 ± 1.24	*32.50 ± 1.29	n = 8
BLOOD SUGAR mg %	89.80 ± 2.78	##53.33 ± 1.66	181.41 ± 4.98	#116.81 ± 3.13	n = 10
LIVER GLYCOGEN mg/gm tissue	2.405 ± 0.067	2.465 ± 0.081	•0.483 ± 0.022	1.897 ± 0.117	n = 7

INSULIN :- In comparison with Epinephrine* group $p < 0.001$

In comparison with Saline** group $p < 0.02$

BLOOD SUGAR :- In comparison with Epinephrine# group $p < 0.001$

In comparison with Saline## group $p < 0.001$

GLYCOGEN :- In comparison with saline group • $p < 0.001$

(Results expressed as Mean ± S.E.M) (student's unpaired 't' test)

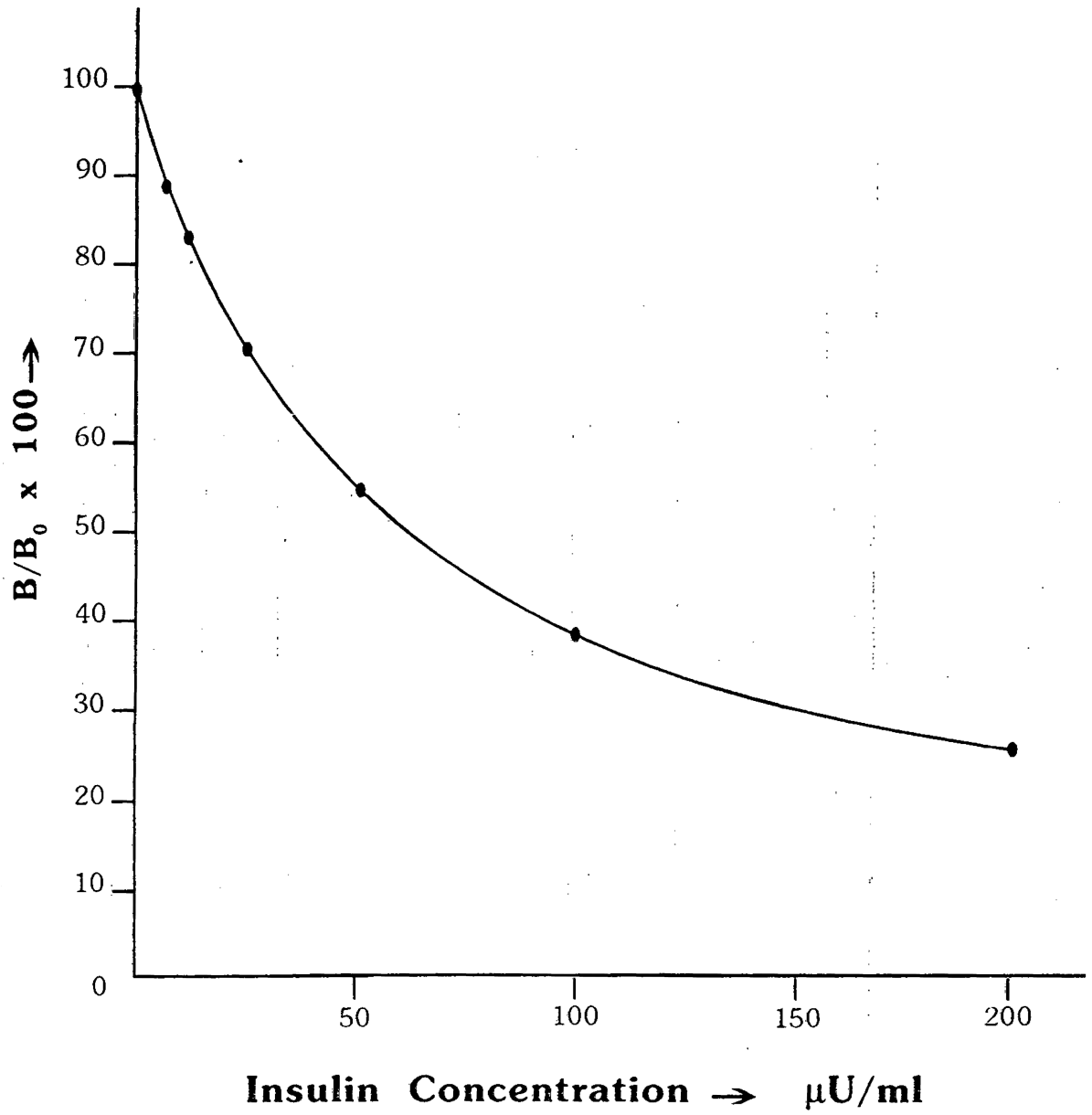

Fig. 13 : Insulin Standard Curve

control values of 16.56 ± 1.41 m units/ml ($p<0.02$) suggesting that *B.P. ext.* causes insulin secretion.

In normal mice, epinephrine caused a marked hyperglycaemia, which in turn gave rise to slight elevation of serum insulin level i.e. 21.61 ± 1.24 m units/ml from the control values.

In *B. pertussis* pretreated mice, however significant lowering of blood sugar was observed along with an increase in the insulin levels 32.5 ± 1.29 m units/ml (± S.E.M, n = 8) indicating that the injection of epinephrine did augment rather than prevent insulin secretion.

The liver glycogen values were also assayed in the above similar groups. The *B.P. ext.* treated group had the glycogen value close to the control values i.e. 2.465 ± .081 mg/gm tissue. Administration of epinephrine caused glycogenolysis and thus glycogen values reduced from the control value of 2.405 ± 0.067 to 0.483 ± 0.022 mg/gm tissue (± S.E.M, n = 7). Pretreatment with *B.P. ext.* slightly lowered the glycogen values to 1.897 ± 0.117 mg/gm tissue with no increase in *phosphorylase a* activity. It thus shows that *B.P. ext.* prevents the massive breakdown of glycogen by epinephrine.

Discussion

B. pertussis extract is known to cause hypoglycaemia at the end of 6 hours in normal mice. It was also observed that the partially purified extract (1 mg/kg body weight) stimulated insulin secretion from pancreas. It was found to be a protein with minute amounts of carbohydrates (1.5 mg%) and lipids (400 µg%). There were also no toxic symptoms or death of the animals even upto a period of 4 weeks with the dose level studied.

This extract prepared from *Bordetella pertussis* (strain no 18-323) on intravenous administration showed a maximum and significant lowering of blood sugar at the end of 6 hours. This is in sharp contrast to that from the previously used vaccine strain (strain no 25-525) which showed a comparable lowering only at the end of 72 hours, for the identical dose administered[164].

Epinephrine administration (i.p.) caused significant hyperglycaemia in mice as early as 30 minutes. To examine whether the *B.P. ext.* could reverse epinephrine induced hyperglycaemia, it was administered to *B.P. ext.* pretreated mice. The extract caused a blood sugar lowering of 35.61% at the end of 30 minutes ($p<0.001$).

Since the *B.P. ext.* caused a maximum blood sugar lowering at the end of 6 hours, it was administered in such a way that it's maximum action (6 hours) coincided with the peak action of epinephrine, for the various parameters studied.

Biochemical Parameters :-

As confirmed by the results of this study, the extract on prior treatment demonstrated a significant lowering effect in liver against epinephrine induced glycogenolysis and gluconeogenesis. The parameters studied with respect to the above two mechanisms were aspT, alaT, serine-dehydratase, PEP-CK, *phosphorylase a*, glycogen and serum insulin levels.

The levels of enzyme aspT, alaT, serine dehydratase, PEP-CK, *phosphorylase a* and glycogen were studied in mice liver. For enzyme assay, the liver was homogenized in 5 or 10 volumes of the buffer used for that particular assay. The homogenate was centrifuged at 20,000 r.p.m. for 20 minutes at 5°C and the clear supernatent was used to assay different parameters. The enzymes serine dehydratase, PEP-CK and *phosphorylase a* are found exclusively in the cytosol in mouse liver. AspT is of cytosolic as well as mitochondrial origin and alaT predominates in the cytosolic fraction. Triton X 100 treatment was employed to extract the mitochondrial enzymes in to the Cytosol.

Aspartate transaminase :-

Total cytosolic and mitochondrial enzymes were estimated. 0.5% Triton X 100 was used to extract the mitochondrial enzyme, since 1% Triton X 100 was found to inhibit the enzyme activity[176].

On epinephrine administration aspT levels were lowered initially at the end of 1 hr and then increased at the end of two hours. The initial decrease in the enzyme level can be explained due to the hyperglycaemia which in turn causes insulin secretion. Insulin is known to inhibit hepatic glucose production[185]. This insulin causes a rapid utilization of glucose by stimulating the glycolytic and TCA pathway. The increased concentration of TCA pathway metabolites such as succinate, maleate have been reported to be the competitive inhibitors of the enzyme aspT[78]. Therefore it appears that the lowering of aspT at the end of one hour could be due to the overall effect of insulin and hyper-glycaemia.

The B.P. ext. lowered the enzyme AspT significantly in epinephrine treated mice (16.94% lowering) ($p<0.001$) and 21.9% in normal mice.

Alanine transaminase :-

Enzyme alanine transaminase is found (85%) in the cytosolic fraction[186]. AlaT was not found to increase after epinephrine administration. It can be explained as follows : The Km for liver cytosolic alaT for alanine is 34 mM, which is very high[186]. Hence this low activity of enzyme could be attributed to low alanine concentration in the liver cytosol. The enzyme has an $t^1/_2$ life of 3 - 3.5 days, hence no rapid increase is observed in enzyme levels[187]. The enzyme is observed to increase only after 48 hours of fasting. Also

the release of amino-acid alanine from muscles is observed only after 48 hours of fasting, which can then be taken up by the liver for pyruvate formation[188]. This explains the finding of low alaT levels in our studies. Treatment with prednisolone 2.0 mg/day increases the enzyme 1.7 folds after 24 hours with $t^1/_2$ = 1.2 days[86]. This shows that the enzyme levels increase only slowly after hormone treatment.

Serine dehydratase :-

Rat and guinea pig liver is rich in serine-dehydratase and in these species conversion of serine to pyruvate by the enzyme is of considerable significance. In many other vertebrates including humans, the evidence suggests that serine is degraded to glycine and to 5, 10 - methylenetetrahydrofolate[88].

Epinephrine caused a 1.5 fold increase at the end of one hour *B.P. ext.* lowered the levels of the enzyme serine dehydratase significantly by 40% in the epinephrine treated mice ($p<0.001$) as well as by 31.71% in the normal mice. It may be mentioned that the km of the enzyme for serine is 0.91 mM with $t^1/_2$ of $5^1/_2$ hours[189, 190].

Phosphoenol Pyruvate Carboxykinase :-

PEP-CK plays an essential role in gluconeogenesis utilizing three or four carbon precursors. The cytosolic enzyme predominates in rats. The enzyme is responsive to dietary and hormonal changes. The

level of the enzyme is raised by fasting and diabetes and by administration of glucagon, epinephrine, nor-epinephrine, gluco-corticoids, and tryptophan, when administered to intact animals[73].

Epinephrine increased the enzyme PEP-CK 1.5 folds at the end of 2 hours. However on pre treatment with B.P. ext. there was 38.3% lowering in epinephrine treated mice, which was highly significant ($p<.001$). In normal *B.P. ext* treated mice there was a 22% lowering which was also significant ($p<.001$).

Phosphorylase a :-

Phosphorylase a estimation involves incorporation of sodium fluoride (NaF) in the homogenate in order to inhibit the enzyme phosphorylase phosphatase which converts the active enzyme *phosphorylase a* to *phosphorylase b*[191].

On epinephrine administration phosphorylase a was found to increase at the end of 12.5 minutes. The liver glycogen levels was also lowered significantly ($p<.001$). The increase in glucose, subsequent to glycogen breakdown, reached a peak level at the end of 30 minutes. *B.P. ext* significantly prevented the increase in *phosphorylase a* at 12.5 minutes in epinephrine treated mice, although slight lowering in glycogen values were also obtained. Saline and *B.P. ext* *phosphorylase a* levels and glycogen values remain similar to each other.

Thus *B.P. ext.* prevented the stimulation of *phosphorylase a*. Hence the glycogen values did not change and the blood sugar remained low.

It has been reported that *phosphorylase b* is not completely inactive in most assay conditions *in vitro*, although it is certainly inactive in the liver *in vivo*[60]. However the significant changes observed in the enzyme levels in our studies could be attributed only to *phosphorylase a*.

Insulin :-

B.P. ext. treatment was found to significantly increase insulin secretion in epinephrine treated mice as well as normal mice. The mechanism of increased secretion of insulin could be explained as follows :-

Epinephrine binds to two types of adrenergic receptors α and β present on the cell-membrane. The receptors are sub-divided into α_1, α_2, β_1 and β_2 types respectively. Response of any cells to these amines, depend on the proportion of α and β receptors on that particular cell. Pancreatic β islet shows a predominance of α_2 adr type of receptors as compared to β_2 adr receptors. Pancreatic secretion of insulin is promoted by stimulation of adr β_2 receptors and inhibited by stimulation of the α_2 receptor in the islets, depending upon the physiological conditions[192]. Normally epinephrine binds to

the α_2 - receptors of pancreatic β cell and inhibits insulin secretion, though the hyper-glycaemia inturn gives slight elevation in blood insulin level[193]. Epinephrine administration in *B.P ext.* pretreated mice shows increase in the level of insulin suggesting that the β receptor mediated insulin secretion by epinephrine was favoured in the *B. Pertussis* treated mice.

The above results are similar to that reported for *pertussis* vaccine, which state that the β receptor mediated insulin secretion is favoured in *pertussis* sensitized mice[193].

From our studies it is seen that *B.P. ext.* lowers gluconeogenesis as well as glycogenolysis in mice. This effect could be attributed to the insulin secreted on treatment with the extract.

To study whether the extract has any independent action, it was administered to mice which were made severely diabetic by alloxan at a dose of 75 mg/kg b.wt[174]. The high blood sugar levels, mortality, wt loss and negligible levels of serum insulin confirmed the animals to be severely diabetic. Subsequent to *B.P. ext.* treatment it was observed that liver gluconeogeneic enzymes aspT, serine dehydratase and PEP-CK were lowered significantly. Thus the *B.P. ext.* could have an independent action.

Antioxidant and glycolytic effects of the extract :-

Studies in our laboratory show that *B.P. ext.* significantly restored the levels of anti-oxidant enzymes super-oxide dismutase, catalase, glutathione peroxidase and glutathione reductase, which were otherwise severely depleted by alloxan[166].

B.P. ext. was found to stimulate liver glucose-6-phosphate dehydrogenase (G6PD) and increase glucose utilisation in both normal and alloxan diabetic mice[164, 165]. The *ext.* caused a 7 fold increase in the level of G6PD which is the main generator of NADPH in the HMP shunt pathway, which can be used for biosynthetic processes such as fatty acid synthesis and pentoses for nucleic acid and nucleic acid biosynthesis. The reactions catalysed by the 3 kinases, Glucokinase, phospho fructo kinase and pyruvate kinase, are of crtitical importance, since their prevalence over the opposing gluconeogeneic enzymes will favour glycolysis. *B.P. ext.* showed 2 to 3 fold increase in the values of all 3 liver kinases - glucokinase, phosphofructo kinase and pyruvate kinase, thus significantly favouring glycolysis in 18 hours fasted treated mice[165].

Reversal of Scorpion venom toxicity by *B.P.* extract :-

In our laboratory, some preliminary studies were carried out on venom from the red scorpion *Buthus tamulus*. The venom causes the release of a large amount of catecholamines[194] which mediated

glycogenolysis and gluconeogenesis in liver along with lipolysis, increase in potassium levels and inhibition of insulin secretion[195].

At the dose level of 1.5 mg/kg body weight (i.p.) in mice, maximum hyperglycaemia (250 ± 8.49 mg%) was found at the end of 45 minutes after venom administration. Pretreatment of *B.P. ext.* 6 hours before scorpion-venom administration significantly prevented the hyperglycaemia (Bl. Sugar 101 ± 1.25 mg%) (± S.E.M, n = 5) ($p<0.001$). The various sideffects such as hyper-excitability, profuse sweating, excessive salivation and nasal secretion, dilation of pupils of the eye and pilo-erection were prevented by the pretreatment with the ext.

The above observation supports the work done by Murthy[196] who observed several rhythmic changes, conduction defects, infarction like pattern, ECG abnormalities and many other effects such as hyperglycaemia, reduced insulin secretion and glycogen depletion in heart, liver and skeletal muscle within 20-30 minutes of scorpion envenomation in dogs. 10 units of crystalline insulin given (i.v.) corrected the arrhythmia, conduction defects and other ECG abnormalities, increased glycogenesis in liver, cardiac and skeletal muscles.

It is reported that the catecholamines released during autonomic storm in scorpion envenomation[194] are responsible for suppressing insulin secretion, which results in glycogenolysis and lipolysis. Administration of insulin corrects this effect.

It thus becomes clear that *B. pertussis* ext. could probably have reversed some of the effect of the scorpion-venom through its ability to cause insulin secretion.

Receptor mediated effects of epinephrine :-

Epinephrine exerts major effects on carbohydrate metabolism. In liver it promotes hepatic glucose output by activating glycogenolysis and acclerating gluconeogenesis, while simultaneously inhibiting glycogen synthesis[59]. Epinephrine exerts its effects on different mechanism through its adrenergic receptors. There are two different types of adr receptor α and β, which are subdivided into α_1, α_2, β_1 and β_2 sub-types. Proportion of these receptors differs in different cell types, which result in different characteristic changes in different tissues. The responsiveness of peripheral effector tissue to adr stimulation may be modified substantially by alteration in temperature, chemical composition of the plasma and circulating levels of different hormones[2]. These changes in responsiveness may originate at :- (i) the adr nerve terminal where variety of factors influence amount of neurotransmitter release. (ii) the adr receptor level where alteration in receptor numbers may occur. (iii) post receptor sites so that relationship between receptor occupancy and physiological response is modified[2].

Earlier studies by Sutherland, Rall and coworkers were carried out in cat, dog and rabbit liver where β receptors predominate[197].

They had reported that interaction of epinephrine with the β2- adr receptor results in glycogen breakdown through the classical cAMP cascade.

However according to the recent reports, rat liver plasma-membrane contains adr receptors predominantly of α sub type (80% of the total α adr receptor) and adrenergic control of *phosphorylase a* in rat hepatocytes is mediated via α_1 -adr. receptors[198]. In human liver α and β– adr. receptors induce metabolic responses[2]. Stimulation of α_1-adr. receptor causes mobilisation of intracellular Ca^{2+} from endoplasmic stores. This increase in intracellular calcium is currently thought to result from activation of phospholipase C(β) isoform. This results in generation of 2 messengers - DAG and inositol-I, 4, 5-trisphosphate (IP_3). IP_3 stimulates the release of Ca^{2+} from intracellular stores via a specific receptor, while DAG is a potent activator of protein kinase C. It mediates phosphorylation of proteins. The mobilised Ca^{2+} causes activation of phosphorylase kinase, which converts *phosphorylase b* to *phosphorylase a*, aided by inhibition of glycogen synthase[7]. This explains the stimulating effect of epinephrine on glycogen phosphorylase in mouse liver.

It also suggests that epinephrine stimulated gluconeogenesis from lactate, pyruvate or alanine by a mechanism apparently unrelated to cAMP[22, 51]. Amino-acid uptake into the liver and perhaps lactate entry also, is augmented by α– agonist[99, 199]. thus

increasing the availability of substrates for gluconeogenesis. Glucose 6-phosphatase, Hexose-diphosphatase[51], PEP-CK are also stimulated after epinephrine infusion[101, 200].

Mechanism of action of *B.P.* extract :-

As it appears *B pertussis* extract acts through i) It's ability to cause insulin secretion and ii) it's independent action of liver.

1) Insulin Secretion :-

It has been reported that several agents inhibit insulin secretion. Among them are α_2 -adr. Agonist such as epinephrine, norepinephrine and clonidine, the peptides galanin and somatostatin and the prostaglandins[201]. They act on the β islet cell by preventing Ca^{2+} mobilisation. They exert their effect individually by inhibiting voltage dependent Ca^{2+} channels, L-type voltage dependent channels and adenylate cyclase. The above functions are blocked by prior treatment of animal islet or β cells with pertussis toxin[201]. All of these mechanisms have been described as pertussis-toxin (PTx) sensitive, with the implication that G proteins of Gi/Go types are mediating the several known inhibiting functions[201]. PTx treatment causes ADP ribosylation of islet G-protein α sub-units at different rates ($\alpha_0 > \alpha_{i2} > \alpha_{i3}$), a finding that allows for the differential effect of PTx on the action of different hormones[201].

Komatsu,; and McDermott, have reported a complete reversal of nor-epinephrine induced inhibition of insulin secretion in β cells between 48-72 hours on PTx pretreatment[201]. Such pretreated cells, when treated with nor-epinephrine as well as 16.7 mM glucose showed a 3.69 fold increase in insulin secretion at the end of 72 hours[201]. In our studies in mice pretreatment with *B.P. ext.* showed a 1.5 fold increase in insulin secretion as early as 6 hours after epinephrine administration without glucose infusion.

Thus in pancreatic β cell, PTx appears to block the α-adr receptor by ADP-ribosylation and thus causes a slow influx of Ca^{2+}. This inturn activates adenylate cyclase and the increased cAMP (and Ca^{2+} levels) stimulate secretion of insulin (hyper insulinemia)[148]. A similar mechanism may be attributed to *B.P. ext.* used in our studies.

2) <u>Independent action on liver</u> :-

Although liver cell plasma membrane possess α_1-, α_2- and β-adr. binding sites, it is generally agreed that in mature male rats, the control of hepatic glycogenolysis by catecholamine is mediated largely by α_1- while β receptor exerts little, if any regulation of cAMP levels[202].

Thus, majority of hormones epinephrine, nor-epinephrine, EGF and vasopressin cause PIP_2 breakdown in the liver cells and causes calcium mobilisation[203, 204]. PTx pretreatment prevents the PIP_2

breakdown by various hormones thereby inhibiting the Ca^{2+} mobilisation in the hepatocytes.

Similar mechanism may be attributed to B.P. ext. used in our studies,

With regard to metformin, recent studies shown that the frequency of α_1-adr agonist induced oscillation of free intracellular Ca^{2+} was lowered by treatment with metformin. It thus exerts anti-diabetic effect by affecting calcium mobilisation[205].

It can thus be concluded that the *B. pertussis ext.* inhibits hepatic glucose output by inhibiting glycogenolysis and gluconeogenesis. It could achieve this through insulin secretion which inhibits hepatic glucose output and also possibly through its individual action on phosphoinositol breakdown and Ca^{2+} mobilisation in hepatocytes as explained for PTx.

Summary and Conclusion

1) The protein extract from *Bordetella pertussis* cells (Strain no. 18-323) caused significant hypoglycaemia at the end of 6 hours in mice and significantly attenuated epinephrine induced hyperglycaemia.

2) *B.P. ext.* significantly lowered epinephrine induced hyperglycaemia by preventing the increase of the enzyme *phosphorylase a* in liver and by increasing glucose utilization.

3) The ext. significantly lowered the levels of gluconeogeneic enzymes such as aspartate transaminase, serine dehydratase and phospho-enol pyruvate carboxy kinase in normal mice.

4) It significantly also lowered the levels of enzymes aspartate transaminase, serine dehydratase and phospho-enol pyruvate carboxy kinase in severely affected alloxan diabetic mice. It suggests an independent action of the extract on gluconeogenesis.

5) The *B.P.* ext. caused insulin secretion at the end of 6 hours. Epinephrine normally inhibits insulin secretion but pretreatment with the *B.P. ext.* favoured significant secretion of insulin. Thus the ext. did augment rather than prevent insulin secretion.

6) The unique effect of the extract on carbohydrate metabolism appears to be the combined effect of :

a) its ability to cause insulin release from the pancreas.

b) and possibly its unique independent action on liver.

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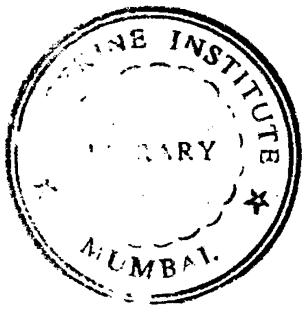

Synopsis

A SYNOPSIS

OF THE THESIS TO BE SUBMITTED TO

THE UNIVERSITY OF BOMBAY

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN BIOCHEMISTRY

TITLE OF THE THESIS	:	STUDIES ON THE EFFECT OF *BORDETELLA PERTUSSIS* PREPARATION ON HYPERGLYCAEMIA AND ASSOCIATED RESPONSES INDUCED BY CATECHOLAMINES
NAME OF THE CANDIDATE	:	Mrs. SMITA V. CHITNIS
NAME AND DESIGNATION OF THE GUIDE	:	Dr. P. RAMAKRISHNAN, Chief, Department of Biochemistry, Aditya Pediatric Research Laboratory, B. J. Wadia Hospital for Children, Parel, Bombay 400 012. Former Head, Department of Biochemistry Haffkine Institute, Parel, Bombay 400 012.
PLACE OF RESEARCH	:	Department of Biochemistry Haffkine Institute, Parel, Bombay 400 012.
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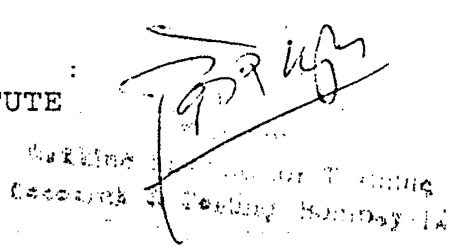


INTRODUCTION

Among the catecholamines, epinephrine exerts important influences on metabolism. The hormone is released under physiological stress such as hypoxia, hypotension, cold, fatigue and shock. It serves as an emergency hormone by rapidly providing fatty acid by lipolysis which are the primary fuel for muscle action. It is also known to mobilize glucose both by increasing glycogenolysis and gluconeogenesis in the liver and by decreasing glucose uptake in the muscle. Epinephrine decreases insulin level thereby preventing glucose from being taken up by peripheral tissues thus preserving it for the Central Nervous System[1]. Further, it also enhances secretion of glucagon from the α cells of pancreas. The increased glycogen breakdown in muscle leads to increase in blood lactate, which is taken up by the liver to synthesize glucose through reversal of glycolysis.

Scorpion venom such as, the one from the Red scorpion *Buthus tamulus* is known to act through release of large amount of catecholamines[2]. Their toxic manifestations include arterial hypertension, cardiovascular abnormalities, pulmonary edema and certain biochemical effects which include hyperglycemia, lipolysis metabolic acidosis, increased potassium levels and inhibition of Insulin secretion.

Cell extract of *Bordetella pertussis*, an aerobic gram negative coccobacillary organism was reported to cause hypoglycemia in

normal mice[3]. Whole cells or extracts of *Bordetella pertussis*, have been reported to significantly reduce the hyperglycemia in alloxan diabetic mice,in addition to increasing tolerance to glucose[4].

The *B. Pertussis* extract (*B.P.* ext.) reversed thediabetogenic effect of alloxan by increasing the rate of utilization of glucose and by significantly restoring the antioxidant enzymes to their normal values, which were otherwise depleted by alloxan[5].

B.P. ext is known to cause an increase in the levels of glycolytic enzymes from mouse liver[6].

However, The effect of *B. P. ext.* onvital physiological mechanisms such as glycogenolysis, gluconeogenesis is not known, particularly in view of the fact that it reverses the diabetogenic effects of alloxan.

Since epinephrine is known to stimulate the above mechanisms, it was decided to study the effect of *B. P. ext.* on the metabolic changes induced by epinephrine.

AIMS AND OBJECTIVES

To study the effect of *B. P. ext.* in epinephrine treated mice and elucidate its mechanism of action. This required the assessment of two mechanisms, namely glycogenolysis and gluconeogenesis in the liver, apart from estimation of blood sugar and serum Insulin levels.

IN LIVER

I. (1) **Glycogenolysis** : It is a CAMP mediated process, which through activation of enzyme cascade leads to formation of glucose. The parameters to be studied include a) **Glycogen** which is the main storage polysaccharide in the animal cell.

b) **Phosphorylase a** which catalyses the breakdown of glycogen to glucose-1-phosphate from which glucose-6-phosphate and then free blood glucose are formed.

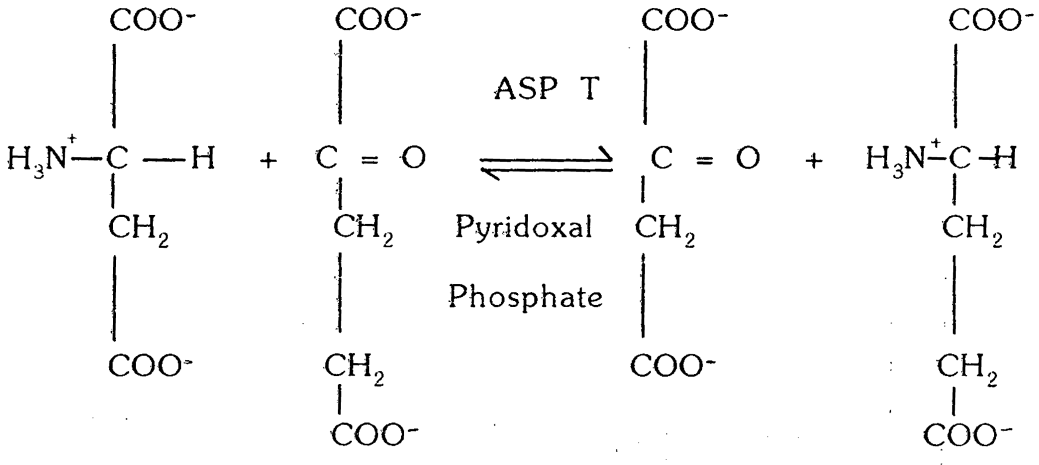
$$(\alpha\text{-}1,4\text{-Glucosyl})_n + P_i \xrightarrow{\textit{Phosphorylase a}} (\alpha\text{-}1,4\text{-Glucosyl})_{n-1} + \alpha\text{-D-Glucose-1-P}$$

Glycogen

Glucose-1-phosphate

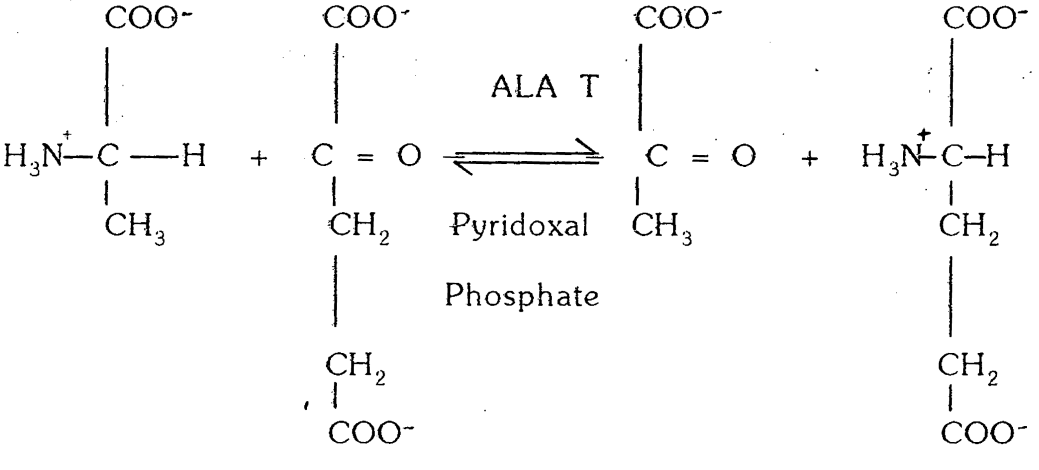
(2) **Gluconeogenesis** : This process involves formation of glucose from non-carbohydrate sources. The enzymes to be studied include:

a) **Aspartate transaminase** : It is a gluconeogeneic enzyme which converts the amino-acid, L-aspartate and 2-oxo-glutarate to yield oxaloacetate which can be converted to glucose through reversal of glycolysis.



L- Aspartic + α Ketoglutarate ⇌ Oxaloacetate + L-Glutamate Acid

b) **Alanine transaminase** : It is a gluconeogeneic enzyme which converts the amino acid L-alanine and 2-oxoglutarate to yield pyruvate.



L-alanine + α Ketoglutarate ⇌ Pyruvate + L-Glutamate

c) **Serine dehydratase** : It is a pyridoxal phosphate containing enzyme which converts the amino acid L-serine through elimination of water and hydolytic loss of ammonia to yield pyruvate.

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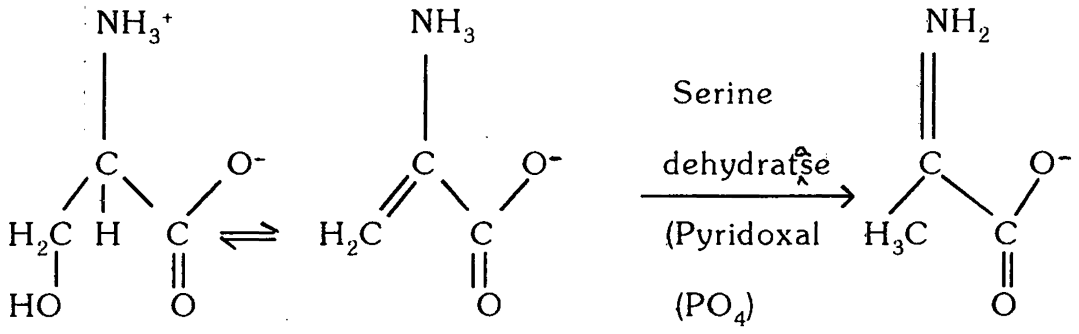
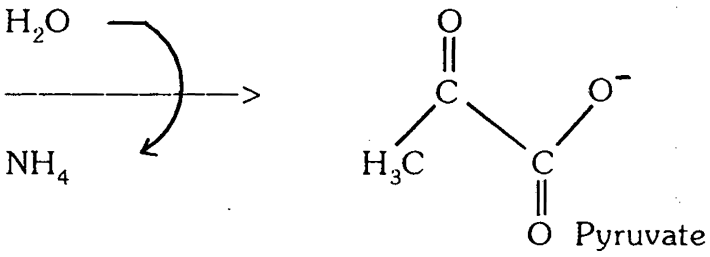
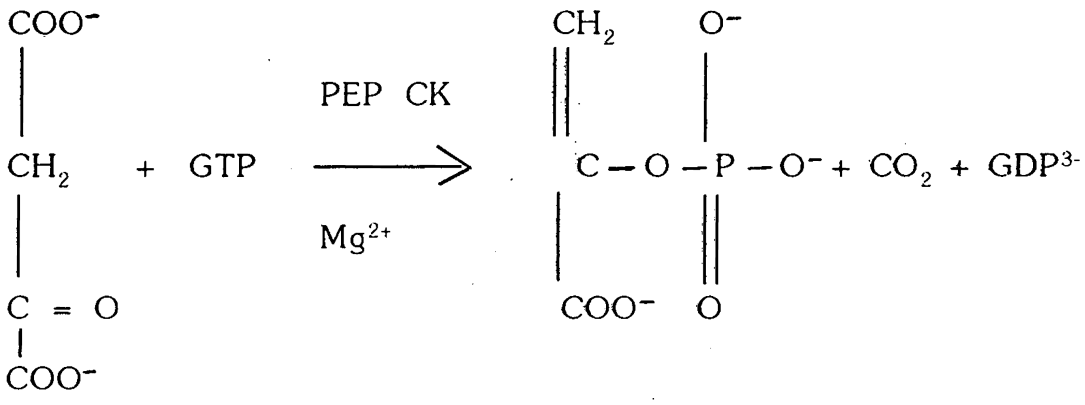


L- Serine



d) **Phosphoenol pyruvate carboxykinase** : This is the rate determining enzyme in gluconeogenesis. It converts the metabolite oxaloacetate to phosphoenol pyruvate.



Oxaloacetate Phosphoenol pyruvate

All these parameters were assayed in mice liver. The levels were studied in epinephrine treated mice as well as in animals pretreated with *B. P ext.*. The enzyme levels were also studied in alloxan diabetic mice and in alloxan diabetic mice treated with *B. P. ext.* This was in

order to examine whether the *B. P. ext.* has an independent action.

II. **To estimate serum insulin levels**:

As epinephrine is known to cause hyperglycaemia and *B. P. ext.* to lower blood sugar levels, it was thought to estimate blood sugar and insulin levels in different groups.

III. To explain the reversal of the above mechanisms by *B. P. ext.*

MATERIALS AND METHODS

(1) *Cell extraction and purification* : *B. Pertussis* strain No. 18323, a standard challenge strain was obtained from the Haffkine Bio-pharmaceutical corporation Ltd. (HBPCL), Bombay. It was cultivated using the facilities in the Bacteriology Department of Haffkine Institute and Bacterial Vaccines Department, HBPCL. The formaldehyde treated 48 hours cell culture was adjusted to pH 4.5 and extracted using a buffered enzyme preparation consisting of Lysozyme, DNase, RNase[7]. This leads to selective solubilization of outer membrane proteins. The final extract was dialysed and concentrated. Protein concentration was determined by the method of Lowry *et al*[8].

(2) **Animal Model** : Male albino mice (24-26 gms body weight) Kasauli strain supplied by HBPCL, Bombay was acclimatized to laboratory conditions and were given food and water ad libitum. They were fasted for 18 hrs prior to each assay. To study the effect of *B. P. ext* in epinephrine treated mice the animals were divided into four groups:

a) **Normal Saline control** : 0.1 ml saline was injected intravenously and parameters assayed.

b) ***B. Pertussis extract* group** : It was given at the dose of 1mg/kg body weight intravenously and the parameters estimated at the end of 6 hours.

c) **Epinephrine treated group** : Epinephrine was given at the dose of 0.4 mg/kg body weight intraperitoneally and parameters estimated at the end of different time intervals[9].

d) ***B. Pertussis* + epinephrine group** : *B. P. ext.* was administered in such a way that its maximum action (6 hours) coincided with the peak action of epinephrine, for the different parameters studied.

To study the independent effect of *B. P. ext*, the animals were divided into two groups -

i) **Alloxan (48 hours) group** : Alloxan was injected intravenously at the lethal dose of 75mg/kg body weight and parameters assayed at the end of 48 hours.

ii) **Alloxan + *B. P. ext.* group** : The alloxan diabetic mice were injected with *B. P. ext.* at the end of 42 hours and assay was performed at the end of 48 hours.

(3) **Dose response and Time rate studies** :

The optimum time and dose required to induce hyperglycaemia by epinephrine and to induce significant hypoglycaemia by *B. P. ext.*

a) **Normal Saline control** : 0.1 ml saline was injected intravenously and parameters assayed.

b) ***B. Pertussis extract* group** : It was given at the dose of 1mg/kg body weight intravenously and the parameters estimated at the end of 6 hours.

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The optimum time and dose required to induce hyperglycaemia by epinephrine and to induce significant hypoglycaemia by *B. P. ext.*

were established in mice. 50 µl of blood was drawn from the orbitosinus and Blood sugar was estimated by o-toluidine method [10].

(4) **Studies on enzyme activities** :

The enzyme parameters were assayed in mice liver. All procedures were carried out at 4°C. Fasted mice (18 hours) were decapitated, liver excised immediately and washed in ice-cold saline. It was homogenised in 10 volume (w/v) of respective buffer required for that particular assay. The homogenate was centrifuged at 20,000 RPM for 20 minutes at 5°C and the clear supernatent assayed for following parameters colorimetrically or spectrophotometrically.

i) **Aspartate transaminase amd Alanine transaminase** : The liver tissue was homogenised in 0.25 M sucrose + 1 mM EDTA for 60 seconds to give a 10% homogenate. The homogenate was treated with Triton X 100 at a concentration of 0.5 % and the tubes were kept at 0°C/30 minutes. The homogenate was then centrifuged at 15,000 rpm for 20 minutes and the supernatent was assayed for aspartate transaminase by the method of Rej, R.and Horder, M[11]. and assayed for alanine transaminase by the method of Horder, M. and Rej, R[12].

ii) **Serine dehydratase** : It was estimated by the method of Suda, M. and Nakagawa, H[13].

iii) **Phosphoenol pyruvate carboxykinase** : It wasestimated by the method of Lane *et. al*[14].

iv) ***Phosphorylase a*** : It was estimated by the method described by Bergmeyer[15].

v) **Liver glycogen** : It was estimated by the combined method of Trevelyan W.E *et al* and Seifter *et al*[16,17]

Protein was estimated by the method of Lowry et al[8] and the enzyme activity expressed as units/mg protein. Chemicals used were of Excelar grade or from Sigma.

(5) **Insulin Assay** : Blood was collected from various groups of mice and the serum was seperated.

Serum insulin levels were assayed by Radio-immuno assay (RIA) by the double antibody technique using the kit supplied by Board of Radiation and Isotope Technology , BARC, Bombay, India.

Results were expressed as mean ± S.E.M. and significant was calculated using student's unpaired t test.

RESULTS

1) *B. P. ext* at the dose level of 1 mg/kg body weight (i.v.) lowered blood sugar by 45.72% and 50.38% from normal values at the end of 6 and 12 hours respectively ($p < 0.001$).

2) As shown in the table I, epinephrine at 0.4 mg/kg body weight (i.p.) showed maximum hyperglycaemia at the end of 30 minutes (181.41 ± 4.98 mg%). But this was significantly lowered by administration of the extract before epinephrine (116.81 ± 3.13 mg %) ($p < 0.001$).

TABLE - I

REVERSAL OF EPINEPHRINE INDUCED HYPERGLYCAEMIA IN MICE BY *B. PERTUSSIS* EXTRACT

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	BLOOD SUGAR IN mg %		
GROUP TREATMENT	30 MINUTES	60 MINUTES	90 MINUTES
SALINE	89.88 ± 2.78	93.68 ± 2.55	94.04 ± 3.63
B.PERTUSSIS EXT.	53.33 ± 1.66	55.64 ± 1.58	58.19 ± 1.17
EPINEPHRINE	181.41 ± 4.98	136.22 ± 2.02	109.66 ± 2.27
B.P EXT+EPINEPHRINE	*116.81 ± 3.13	95.04 ± 3.23	96.17 ± 3.06

Results expressed as Mean ± S.E.M., n = 10

In comparison with Epinephrine* group $p < 0.001$ (student's unpaired 't' test)

3) As shown in Table II, administration of *B.P. ext.* lowered liver enzyme Aspartate transaminase significantly at the end of 6 hours. In normal mice, epinephrine caused an increase in the enzyme level at the end of 2 hours (1.24 ± 0.007 u/mg protein). Pretreatment with *B.P. ext* significantly lowered the enzyme to 1.03 ± 0.020 u/mg protein ($p < 0.001$)

In severe alloxan diabetic mice the enzyme increased to 1.43 ± 0.019 u/mg protein at the end of 48 hours. Treatment with *B.P.ext* 6 hours before the estimation of the enzyme in alloxan diabetic group significantly lowered the enzyme to 1.32 ± 0.014. ($p < 0.001$)

4) As shown in Table III, the enzyme alanine transaminase did not increase significantly on epinephrine administration nor did it decrease in the *B. P. ext.* treated group or alloxan treated group.

5) As shown in Table IV, administration of *B. P. ext.* lowered the enzyme Serine dehydratase levels in normal fasted mice ($p < 0.001$). In normal mice epinephrine caused an increase in the level of enzyme at the end of 60 minutes (0.24 ± 0.005 u/mg protein. Pretreatment with *B. P. ext.* significantly lowered the enzyme level to 0.144 ± 0.005 u/mg protein. ($p < 0.001$).

In severe alloxan diabetic mice, the enzyme level increased to 0.23 ± 0.009 u/mg protein at the end of 48 hours which was lowered significantly by administration of *B. P. ext* 6 hours before the estimation of the enzyme, to 0.16 ± 0.011 u/mg protein. ($p < 0.001$).

TABLE - II

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER ASPARTATE TRANSAMINASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U/mg PROTEIN		
GROUP	TIME AFTER EPINEPHRINE ADMINISTRATION		
TREATMENT	1 HOUR	2 HOURS	3 HOURS
SALINE	----	1.037 ± 0.029	----
B.PERTUSSIS EXT.	0.87 ± 0.007	0.81 ± 0.017	0.89 ± 0.022
EPINEPHRINE	0.53 ± 0.017	1.24 ± 0.007	0.88 ± 0.012
B.P EXT+EPINEPHRINE	1.09 ± 0.011	*1.03 ± 0.020	1.06 ± 0.021
ALLOXAN (48 HRS)	1.43 ± 0.019		
ALLOXAN+*B.P EXT.*	**1.32 ± 0.014		

Results expressed as Mean ± S.E.M., n = 7

In comparison with Epinephrine* group $p < 0.001$

In comparison with Alloxan (48 hrs)** group $p < 0.001$ (student's unpaired 't' test)

TABLE - III

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER ALANINE TRANSAMINASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) *B.pertussis ext.* = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U/mg PROTEIN		
GROUP	TIME AFTER EPNEPHRINE ADMINISTRATION		
TREATMENT	1 HOUR	2 HOURS	3 HOURS
SALINE	----	0.38 ± 0.009	----
B.PERTUSSIS EXT.	0.34 ± 0.011	0.33 ± 0.007	0.37 ± 0.016
EPINEPHRINE	0.28 ± 0.008	0.33 ± 0.015	0.28 ± 0.012
B.P EXT+EPINEPHRINE	0.38 ± 0.004	0.36 ± 0.012	0.37 ± 0.008
ALLOXAN (48 HRS)	0.33 ± 0.016		
ALLOXAN+*B.P EXT.*	0.33 ± 0.010		

Results expressed as Mean ± S.E.M., n = 7

Not significant

TABLE -IV

EFFECT OF *B.PERTUSSIS* EXTRACT ON LIVER GLUCONEOGENEIC ENZYME SERINE DEHYDRATASE AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	SPECIFIC ACTIVITY OF ENZYME = U/mg PROTEIN		
GROUP	TIME AFTER EPINEPHRINE ADMINISTRATION		
TREATMENT	30 MINUTES	60 MINUTES	90 MINUTES
SALINE	----	0.164 ± 0.005	----
B.PERTUSSIS EXT.	0.108 ± 0.003	0.112 ± 0.006	0.114 ± 0.007
EPINEPHRINE	0.176 ± 0.003	0.24 ± 0.005	0.14 ± 0.006
B.P EXT+EPINEPHRINE	0.153 ± 0.004	*0.144 ± 0.005	0.124 ± 0.008
ALLOXAN (48 HRS)	0.23 ± 0.009		
ALLOXAN+*B.P EXT.*	**0.16 ± 0.011		

Results expressed as Mean ± S.E.M., n = 7

In comparison with Epinephrine* group $p < 0.001$

In comparison with Alloxan (48 hrs)** group $p < 0.001$ (student's unpaired 't' test)

TABLE - V

EFFECT OF *B.PERTUSSIS* EXTRACT ON SERUM INSULIN LEVELS AFTER ADMINISTRATION OF EPINEPHRINE

Dose :- Epinephrine = 0.4 mg/Kg b. wt (ip) B.pertussis ext. = 1.0 mg/Kg b. wt (iv)

	NORMAL SALINE GROUP	*B.PERTUSSIS* EXTRACT (6 HRS) GROUP	EPINEPHRINE (30 MINUTES)	*B.PERTUSSIS EXT.* + EPINEPHRINE (30 MINUTES)	
INSULIN μ units/ml serum	16.56 ± 1.41	**26.25 ± 3.40	21.61 ± 1.24	*32.50 ± 1.29	n = 8
BLOOD SUGAR mg %	89.80 ± 2.78	##53.33 ± 1.66	181.41 ± 4.98	#116.81 ± 3.13	n = 10

INSULIN :- In comparison with Epinephrine* group $p < 0.001$

In comparison with Saline** group $p < 0.02$

BLOOD SUGAR :- In comparison with Epinephrine# group $p < 0.001$

In comparison with Saline## group $p < 0.001$

(Results expressed as Mean ± S.E.M) (student's unpaired 't' test)

6) It can be seen from Table V, that administration of *B.P.ext* showed increase in the serum insulin levels at the end of 6 hours ($p < 0.02$) suggesting that *B.P.ext.* causes insulin secretion. In normal mice, epinephrine caused a marked hyperglycaemia which in turn gave rise to a slight elevation of blood insulin level. In pertussis sensitized mice, however, significant lowering of blood sugar was observed along with an increase in insulin levels indicating that injection of epinephrine did augment rather than prevent insulin secretion.

7) The effect of *B. P. ext.* on other parameters such as enzyme phosphoenol pyruvate carboxykinase, *phosphorylase a* and glycogen will be discussed in the thesis.

DISCUSSION

B. P. ext. is known to cause hypoglycaemia at the end of 6 hours in normal mice. It was also observed that *B. P. ext.* (1mg/kg body weight) stimulated insulin secretion from pancreas. The *B.P.ext.* was found to be protein with minute amounts of carbohydrate (1.5 mg%) & lipids (400 µg%). These were also no toxic symptoms or death of the animals even upto a period of 4 weeks with the dose level studied.

Epinephrine administration caused significant hyperglycaemia in mice as early as 30 minutes. To examine whether the *B. P. ext.* could reverse epinephrine induced hyperglycaemia, epinephrine was administered in *B. P. ext.* treated mice and it was found that

the extract caused a blood sugar lowering of 35.61% at the end of 30 minutes ($p < 0.001$).

The effect of *B. P. ext.* has been studied on glycolytic enzymes. The *B. P. ext.* significantly increased the levels of glycolytic enzymes suggesting that glucose utilization was favoured by the extract.. The effect of *B. P. ext.* on gluconeogeneic enzyme was studied.

With respect to the enzyme Aspartate transaminase. On epinephrine administration enzyme Aspartate transaminase lowered initially at the end of one hour and then increased at two hours. The initial decrease in the enzyme level can be explained by the hyperglycaemia which in turn caused insulin secretion. Insulin is known to inhibit hepatic glucose production[18].This insulin caused rapid utilization of glucose by stimulating the glycolytic and TCA pathway. The increased concentration of TCA pathway metabolites such as Succinate, Maleate have been reported to be the competetive inhibitors of the enzymes Aspartate transaminase[19]. Therefore it appears that the lowering of Aspartate transaminase at the end of one hour could be the overall effect of insulin and hyperglycaemia. The *B. P. ext.* lowered the enzyme Aspartate tranaminase significantly in normal and epinephrine treated mice.

The enzyme Alanine transaminase was not found to increase after epinephrine administration. It can be explained as follows. The cytosolic alanine transaminase has a Km for alanine of 34 mM which is very high (low activity at lower concentration)[20]. 85% of the enzyme is found to

be in the cytosolic fraction[20]. The enzyme has an t1/2 life of 3-3.5 days hence no rapid increase is observed in enzyme levels.[21] The enzyme is observed to increase after 48 hours of fasting. The release of amino-acid alanine from muscles is observed only after 48 hours fasting which can be taken up by the liver for pyruvate formation.[22] This explains the low levels of enzyme Alanine transaminase. Treatment with prednisolone 2.0mg/day increases the enzyme 1.7 folds after 24 hours with t1/2=1.2 days.[23]

B. P.ext. lowered the levels of enzyme Serine dehydratase significantly by 31.71% in normal as well as 40% in epinephrine treated mice.

With insulin levels, it was found that insulin secretion was significantly enhanced in the *B. P. ext.* pretreated groups. The mechanism of increased secretion of Insulin can be expained as follows :

Epinephrine binds to two types of adrenergic receptor α and β present on the cell membrane. The receptors are subdivided into α_1, α_2, β_1 and β_2 types respectively. Response of any cells to these amines, depends on the proportion of α and β receptors present on that particular cell. Pancreatic secretion of insulin is promoted by stimulation of adrenergic β_2 receptors and inhibited by stimulation of the α_2 receptor in the islets, depending upon the physiological conditions[24]. Normally epinephrine binds to the α_2 receptor of pancreatic β cell and inhibits insulin secretion, though the hyperglycaemia inturn gives slight elevation in blood insulin level[25]. Epinephrine administration in *B. Pertussis* pretreated mice shows increase in the level of insulin suggesting that the β receptor mediated

insulin secretion by epinephrine was favoured in the *B. pertussis* treated mice.

The above results are similar to that reported for Pertussis vaccine which states that β receptor mediated insulin secretion is favoured in Pertussis sensitised mice[25].

Thus,it can be seen that *B. P. ext.* lowers the gluconeogeneic enzymes in mice. Its effect could be attributed to the insulin secreted on treatment with the extract.

To study whether the extract has any independent action, it was administered to mice which were made severely diabetic by treating them with alloxan at the dose of 75 mg/kg body weight. The mortality, weight loss, persistent high blood sugar levels and negligible levels of serum insulin confirmed the animals to be diabetic. They were then treated with *B. P. ext.* It was observed that,liver gluconeogeneic enzymes Aspartate transaminase and Serine dehydratase were lowered significantly by the extract.

Thus it can be said that *B. pertussis* extract significantly reverses the hyperglycaemia as well as other metabolic changes in liver, induced by epinephrine. This coule be due to both through mediation of insulin release as well by an independent action.

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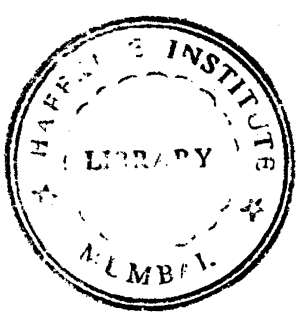

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