Synthesis of Biguanides and Guanamines and Their Effect on Glycogen Phosphorylase a and Steroid Metabolism in Cultured Hepatocytes from Normal and Streptozotocin Induced Diabetic Rats

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow.

By

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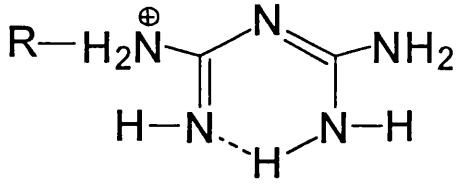
CONTENTS

SUMMARY

Since the introduction of β-phenethylbiguanide (DBI)) as an outstanding blood sugar lowering drug, considerable attention has been directed towards the synthesis of compounds which are in some way related to biguanides as potential anti-diabetic compounds. It may be noted that the biguanides when written in cyclic hydrogen bonded form (I), resemble a triazine-like structure (II). On the basis of the consideration as to whether stabilisation of the biguanide group by ring formation could enhance the anti diabetic activity of these compounds, a series of substituted s-triazine derivatives was synthesised and tested as oral hypoglycaemics in various animal models.



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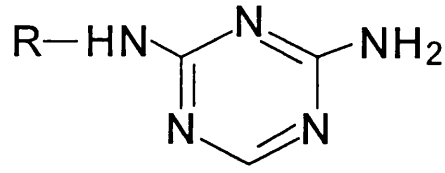


II

Guanamine-like compounds have previously been shown to lower blood glucose levels in normal as well as diabetic rats.

In the present study a series of biguanide and guanamine derivatives were synthesised and tested for their ability to decrease glycogen phosphorylase a in hepatocytes isolated from non- diabetic and streptozotocin-induced (STZ) diabetic rats, as a measure of their insulin-like action. The biguanides such as the phenyl-, benzyl- and phenethyl (phenformin, a therapeutically useful) derivatives were cyclized into guanamines to see the effect of cyclization on the activity of the compound. It was also possible to determine the effect of changing substituents in various positions and to determine the structure-activity relationship of these compounds synthesised for their insulin-like action.

All of the results can be summarised as follows.

(1). In hepatocytes isolated from control animals, (a) all of the synthesised biguanides and guanamines mimic insulin in decreasing glycogen phosphorylase. (b) in the biguanide series, the benzyl and phenyl derivatives were more potent than phenethylbiguanide (phenformin). (c) cyclization of the biguanide to the corresponding 1,2,3-triazines (guanamines) still gives compounds which inhibit the activity of glycogen phosphorylase.

(2). In hepatocytes isolated from diabetic rats, all compounds were seen to increase glycogen phosphorylase a activity in contrast to the inhibition seen in cells from control animals. The most potent compounds were phenethylbiguanide (phenformin) and phenethylguanamine. This effect is opposite to that seen in hepatocytes from control animals and may be compensation for the inhibition of glycogen phosphorylase activity seen in liver from diabetic animals which is, perhaps, due to the low amount of glycogen seen in the liver of these animals. In contrast to the situation in control hepatocytes, diabetic hepatocytes showed the greatest response to phenethylbiguanide and only a moderate response to benzylbiguanide. As in the control animals, however, biguanides and guanamines mimic insulin and thus, the guanamines may prove useful as antidiabetics.

(3). The effect of biguanides and guanamines on glycogen phosphorylase a in the insulin treated diabetic rat was much more like the effect of the compounds on normal rat hepatocytes.

(4). The next series of experiments was performed to try and elucidate the reason for the differences in effects of compounds on glycogen phosphorylase activity in different types of cells by preincubation of normal rat hepatocytes in different concentrations of glucose. A similar structure - activity relationship was observed in control cells preincubated in 30mM glucose as in cells isolated from STZ diabetic rats.

(5) It was observed that all of the compounds mimic insulin in stimulating the activity of glycogen phosphorylase when the diabetic cells are preincubated in 10mM glucose and by decreasing glycogen phosphorylase activity in cells preincubated in 30 mM glucose. This result suggests that cells isolated from STZ diabetic rats are not normal and respond differently to cells isolated from control liver .

(6). The insulin-mimetic effects of phenethylbiguanide and phenethylguanamine in increasing androst-4- ene- 3,17- dione metabolism were examined in order to extend our knowledge of the insulin-like action of biguanides and guanamines from glycogen metabolism to steroid metabolism. Phenethylbiguanide and phenethylguanamine are both able to mimic the effect of insulin in normal rat hepatocytes with respect to androst-4-ene-3,17- dione metabolism. However, the cyclization of phenethylbiguanide to phenethylguanamine gives a compound which is only effective on the cytochrome P450-dependent enzymes (7α- 6β- and 16α- hydroxylases). This possibly indicates that the three cytochrome P450 dependent enzymes are more susceptible than the flavin-dependent enzymes to the triazine moiety in phenethylguanamine.

Overall, biguanides and guanamines have been shown to have significant, direct, insulin-like effects on glycogen phosphorylase a activity and androst-4-ene-3,17-dione metabolism in hepatocytes isolated from control and STZ-diabetic rats. The effectiveness of the compounds depended on the enzyme under study and on the original state of the animal (diabetic or not).

Further investigation is warranted on the usefulness of congeners of the presently used biguanide, phenformin, and on the cyclised biguanides, such as the guanamines and, perhaps, the five-membered structures, the triazoles.

LIST OF FIGURES

INTRODUCTION

1.1. BIGUANIDES.

1.1.1 Historical Background

For more than seventy years it has been known that guanidine itself (Watanabe, 1918) as well as substituted guanidine , diguanidines (Frank *et al.*, 1926; Bischoff, 1929) and biguanides (Slotta & Tschesche, 1928) exhibit blood sugar lowering activity. A large number of compounds have been developed on the basis of the guanidine structure, most of which, however give intolerable side effects. Only a few compounds appear in the narrow range between toxicity and useful drug action and are still used in the treatment of diabetes mellitus. Some of the guanidine derivatives used in the 1920s such as galegine and alkyl biguanides (e.g synthalin A) were found to be too toxic for clinical use and were discontinued as insulin became more widely available (Beckman, 1971; Sterne, 1969). Chlorguanide hydrochloride, introduced as an anti malarial agent in the 1940s, was known to have a weak glucose-lowering effect (Chen & anderson ; 1947). However, it was not until the 1950s, prompted by the introduction of sulfonylureas, that biguanides were re investigated for the treatment of diabetes. In 1957 metformin (Sterne, 1957) and phenformin (Ungar *et al.*, 1957) and in 1958 buformin (Mehnert & Seitz, 1958) were described as agents suitable for the treatment of hyperglycaemia. Structure - activity relationship studies were undertaken with an extensive range of biguanide derivatives, but other suitably active compounds were not found (Beckmann, 1971). Buformin received only limited use, but phenformin was adopted widely in the 1960s and early 1970s (Beckmann, 1971; Mehnert & Haese, 1971). An association with lactic acidosis resulted in withdrawal of phenformin in some countries (Williams & Palmer, 1975; Nattrass & Alberti, 1978), but it is still carefully used in other, either as a monotherapy or in combination with a sulfonylurea. A particular interest has been shown in fixed combination tablets containing phenformin and glyburide (Raptis *et al.*, 1990).

1.1.2 Mechanism of Action.

In spite of intensive research the mechanism of action of biguanides as anti-hyperglycaemics is still a matter of debate (Bailey, 1992). Since the initial step in insulin action is binding to its receptor target cells, the effects of biguanides as hypoglycaemic agents on insulin binding to its receptor have been studied. Biguanides (phenformin and metformin) at concentrations similar to those giving effects *in vivo*, increase insulin receptor binding *in vivo* and *in vitro* (Iwamoto & Goldfine, Holle *et al.*, 1981, Vigneri *et al.*, 1982). Garcia *et al* (1992), using different cell cultures (IM- 9 lymphocytes, H35 hepatoma, cultured breast cells), showed similar results with phenformin. *In vitro* exposure of rat adipose tissue to metformin resulted in an increase in insulin binding (Fantus & Brosseau, 1986). Decrease in insulin binding to the plasma membrane of various target tissues of experimental diabetic animals and patients of different forms of diabetes is well documented (Fussganger *et al.*, 1976; Lockwood *et al.*, 1979). This alteration in insulin binding is essentially due to a change in the number and binding affinity of the insulin receptors located on the plasma membrane of the cells. All forms of diabetes in experimental animals and patients may be broadly classified into two type: insulin deficient and insulin resistant. In insulin deficient diabetes, the number of insulin receptor per cell in target tissues is increased, but the binding affinity of the hormone with it receptor is reduced. In contrast, in insulin resistant diabetes, the number of insulin receptors per cell of the target tissues is reduced but the binding affinity of the hormone with its receptors is increased (De Meyts *et al.*, 1976). Biguanide (metformin) normalises the binding of insulin to its receptor in same diabetic patients (Trischitta *et al.*, 1983).

Whether the site of biguanide action is at the cell membrane and / or at post receptor sites is unclear. Several *in vivo* and *in vitro* studies have pointed out one possible mode of action of the drug at the cell surface, after demonstrating its ability to increase insulin binding to its receptor in various cell types. However, divergent results report the failure of metformin to affect the insulin receptor status in monocytes from

diabetic humans chronically treated with the drug (Prager & Schernthaner, 1983) or in normal insulin target cells such as human (Cigolini *et al.*, 1984) and rat adipocytes (Jacobs *et al.*, 1987) and mouse (Lord *et al.*, 1983) and rat hepatocytes (Alengrin *et al.*, 1987; Wollen & Bailey, 1988).

Alengrin and colleagues (1987) demonstrated that metformin has no direct effect on insulin binding in hepatocytes, indicating that alteration of insulin stimulation of glycogen synthesis in the liver is due to modifications at the post receptor level. Bailey & Puan (1986) showed that metformin increased oxidation of glucose and formation of glycogen from ^{14}C- glucose by streptozotocin diabetic mouse soleus muscle in the presence of maximally stimulating concentrations of insulin. Rossetti *et al* (1990) also reported enhanced muscle glycogen synthetic rate in non- insulin-dependent diabetic rats. An increase in muscle glycogen synthesis by metformin in the presence of insulin has also been described in alloxan diabetic rats (Frayn & Adnitt 1972). Melin *et al* (1990) have suggested that biguanide (metformin) improves type 2 diabetes through an effect at the hepatic level on insulin action by improving insulin - mediated [^{14}C]- glucose incorporation into glycogen in cells isolated from the liver. Biguanide (metformin) increased the active form of glycogen synthase (a form) in both the liver and muscle of diabetic mice.

Reduced gluconeogenesis is frequently considered as a typical biguanide effect (Meyer *et al.*, 1967; Hermann, 1979; Komori *et al.*, 1993). It is generally accepted that biguanides reduce hepatic gluconeogenesis via generation of mitochondrial NADH in hepatocytes (Cook *et al.*, 1973; Ogata *et al.*, 1974). However, hepatic glucose production was increased, presumably due to an increased gluconeogenesis and glucose recycling, in a study performed in Zucker rats (Penicaud *et al.*, 1989). In liver cells high concentrations of metformin ($>10^{-3}M$) reduce basal and glucagon-stimulated gluconeogenesis in the absence of added insulin (Meyer *et al.*, 1967, Cooke *et al.*, 1973). However, therapeutic concentrations of metformin ($10^{-5}M$) act synergistically with physiological concentrations of insulin to reduced gluconeogenesis in isolated rat hepatocytes (Wollen & Bailey, 1988). Metformin in isolated rat

hepatocytes decreased the stimulatory action of glucagon and N, 2-O- dibutyryl- cyclic AMP (db-cAMP) on gluconeogenesis indicating an effect even at a post- adenylate cyclase step (Alengrin *et al.*, 1987). Glucagon stimulated adenylate cyclase activity some 21- fold in liver membranes from obese Zucker rats and treatment of animals with the biguanide (metformin) decreased the ability of glucagon to stimulate this enzyme (Gawler *et al.*, 1988).

It was observed that biguanides inhibited alanine utilisation in the presence of glucagon in isolated perfused livers in normal and diabetic rats, resulting in reduced hepatic gluconeogenesis (Hotta *et al.*, 1991; Komori *et al.*, 1993). This finding suggests that buformin may have reduced hepatic gluconeogenesis from alanine by preventing alanine uptake in liver. Alanine is quantitatively the most important amino acid precursor of gluconeogenesis in the liver (Chochinov *et al.*, 1978).

It has also been observed in *in vitro* studies that metformin increases insulin-stimulated glucose up take into skeletal muscle, fat (Frayn & Adnitt, 1972; Bailey & Puan, 1986; Galsuska *et al.*, 1991; Matthei *et al.*, 1991). and cultured L6 muscle cells of the rat (Hundal *et al.*, 1992). The metformin-induced increase in glucose uptake appears to involve increased translocation of glucose transporters from the microsomal fraction into the plasma membrane, without changing the total cellular pool of transporters (Matthaei *et al.*, 1991; Hundal *et al.*, 1992). In normal fat cells there was increased translocation of both GLUT-4, the main insulin- responsive transporter, and GLUT-1, which is only slightly responsive to insulin (Matthaei et al., 1991). In rat L6 muscle cells, metformin increased translocation of GLUT-1 (but not GLUT-4) into the plasma membrane (Hundal *et al* ., 1992). While it is premature to extrapolate from these *in vitro* models to the *in vivo* situation, the possibility exists that metformin could exert different effects on glucose tranporters in different tissues. Recently, in the investigation of metformin on glucose transport in freshly cultured heart muscle cells from healthy and streptozotocin- diabetic rats, it was shown that the drug caused an approximately 1.6- fold increase in the content of both glucose transporter isoforms, GLUT1 and GLUT4, in the plasma membrane of cardiac myocytes, with a

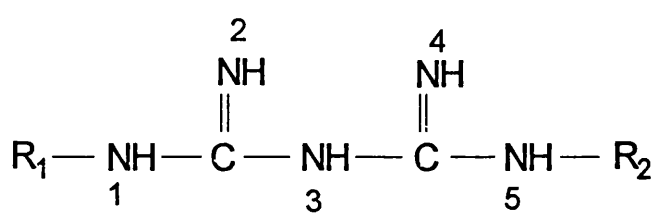
corresponding decrease in the intracellular membrane fraction. In myocytes from diabetic rats, the rate of metformin- activated glucose transport was similar to that of cells from control animals, whereas basal and insulin - stimulated transport were substantially diminished (Fischer *et al.*, 1995). Tissue- specific differences in transporter regulation are known to occur in various physiological and pathological states (Pessin & Bell, 1992). Indeed the intestine, which has a very different glucose tranporter complement from muscle and fat, shows increased glucose utilisation in response to metformin in both the basal and postprandial states (Bailey, 1992). Recently it has been reported that the presence, of insulin is required for metformin to augment glucose utilisation. Thus it is possible that insulin is necessary to activate the cellular processes upon which metformin operates (Bailey & Mynett, 1994).

In an investigation of the effect of metformin and phenformin on the Ca^{2+} signal pathway, it was concluded that the biguanides exert their inhibitory effect on phenylephrine- induced [Ca^{2+}] oscillations by a direct negative interference with the IP_3 sensitive movement of intracellular Ca^{2+} stores (Ubl *et al.*, 1994).

There is , thus, much evidence as to how biguanides exert their effects as anti hyperglycaemic agents but much of it is confusing and contradictory. It is not even clear if biguanides can act alone or need the presence of insulin.

1.1.3. Structure - Activity Relationship of Biguanides.

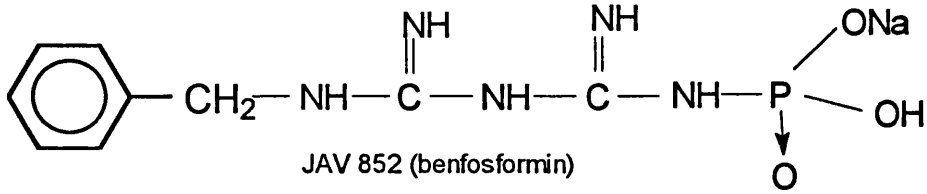
Attempts have been made by Paul and co-worker (1963) to correlate the structure of some series of compounds with their hypoglycaemic actions.



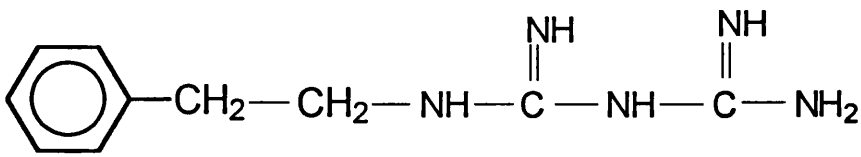
I

The analysis of the results with these compounds based on structure I revealed that when R_1 is phenyl, a little hypoglycaemic activity seems to be developed. Further substitution to the phenyl group at the para position, e.g with, methylsulphonyl, sulphonamido or benzoylsulphonamido compounds, tends to make the compound hyperglycaemic instead of hypoglycaemic. On the other hand, when R_1 is benzyl, the hypoglycaemic activity of the compound is found to be greatly increased. Branching at the $-CH_2-$group of the benzyl moiety with a methyl grouping did not alter the hypoglycaemic response but branching with the next higher homologue, i,e ethyl, markedly decreased the hypoglycaemic activity. when R_1 is ß-phenethyl (phenformin), the resulting compound becomes very active. Substitution at the α-carbon atom of ß-phenethyl by a methyl group reduced the hypoglycaemic activity to a appreciable extent. Further attempts in branching with higher homologues like ethyl, n-propyl, isopropyl, n-butyl or with other substituents like phenyl or benzyl, however, markedly lowered the activity. Substitution of the N5- atom (R_2) of the three potent N1-substituted derivatives (benzyl, -phenyl, ß-phenylethyl) resulted in compounds of lesser activity. This accorded with Shapiro's observation (Shapiro, 1959).

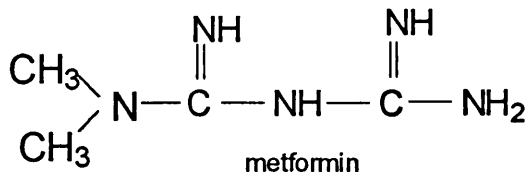
JAV 852 or benfosformin is a phosphorylated biguanide showing powerful anti diabetic properties as well as a low toxicity. Its hypoglycaemic activity, which is more marked in diabetic animals than in normal ones, is comparable to that of phenformin and two to three times as strong as that of metformin. Like the other biguandes, JAV 852 provokes hyperlactacidaemia in diabetic or in non- diabetic animals, but, on the basis of an equal hypcglycaemic effect, the acidosis produced is less than is the case with phenformin or metformin (structure II) (Loiseau *et al.*, 1973).



JAV 852 (benfosformin)



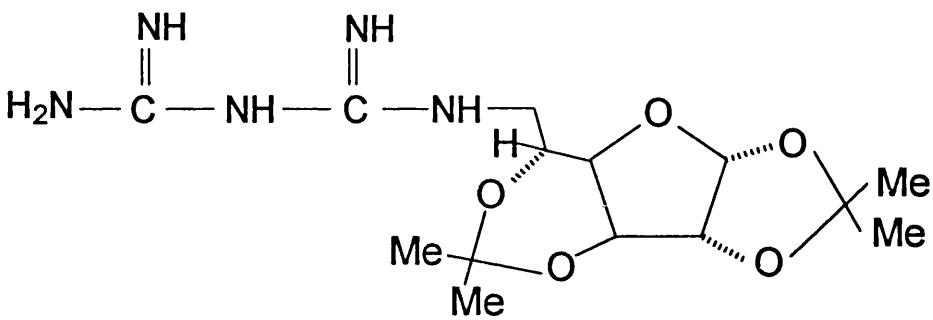
phenformin



metformin

II

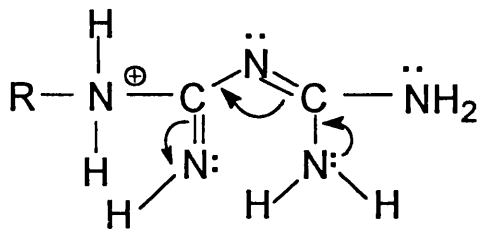
Attaching a biguanide group onto monosaccharides resulted in some compounds where the most active exhibited nearly equivalent hypoglycaemic activity to that phenformin and metformin, such as 6- biguanidino- 1,2: 3,5- bis-o- (1-methylethylidene) -6- deoxy- α- D- glucofuranose (structure III) (Reitz, *et al* 1989).



III

Some aspects of the biological behaviour of biguanides, such as the blood sugar lowering effect and metabolic interactions, have been widely studied (Bechman, 1971; Schafer, 1976). Biguanides form stable co-ordination complexes with divalent cations of oligoelements existing in living organisms. Phenformin and other related oral anti diabetic biguanides form very strong complexes with $Zn^{2+}$and other divalent cations (Ray 1961). This finding suggests that biguanides may produce alterations in the distribution and ratio of divalent cations in biological media (Trijueque, 1981). It

was shown (Haas and Davidoff, 1978) that phenformin administered *in vivo* produced consistent and significant effects on hepatic mitochondrial divalent metal ion content through a more complex mechanism than competitive binding. The structure of this type of complex has been suggested and the one reported by Ray and Saba (1937) is the most widely accepted (structure IV)



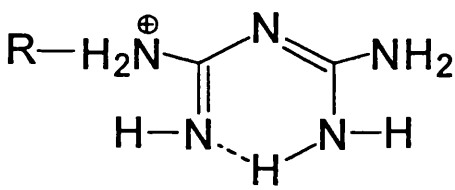
IV

The anti diabetic action of biguanides can be derived from this complex phenomenon. This action must be consistent with some sequence of events such as insulin being stored in β-cells of the pancreas in the form of insoluble insulin-Zinc granules (Lacy 1970) and that biguanides form stronger complexes than insulin does with Zn^{2+}. Formation of such complexes may release insulin from the granules allowing it to enter the systemic circulation. The existence of active electron pairs on some nitrogen atoms in phenformin is inferred and this finding emphasises the ability of biguanides to form hydrogen bridges with other molecular species such as amino acids and proteins, as well as to form co-ordination complexes with zinc and other metallic cations. The anti diabetic action of phenformin and other related biguanides may be explained in terms of competition between these molecules and insulin to co-ordinate cationic oligoelements together with their ability to form hydrogen bonds between the biguanide moiety and insulin itself (Vicente-Pedros *et al.*, 1983). Reduction of the concentration of zinc ions in the plasma has been associated with hypoglycaemia induced by phenformin (Shoka, 1992)

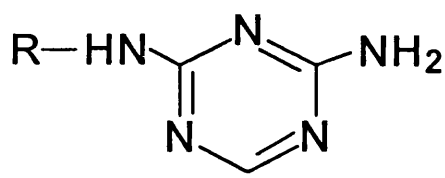
1.2. STUDIES ON RELATED ANTI DIABETIC AGENTS.

Since the introduction of ß-phenethylbiguanide (Ungar *et al*, 1957) (DBI) as an outstanding blood sugar lowering drug, considerable attention has been directed towards the synthesis of compounds which are in some way related to biguanides. It may be noted that the biguanides when written in cyclic hydrogen bonded form (V), seem to represent a triazine - like structure (VI). On the basis of the consideration as to whether stabilisation of the biguanide group by ring formation would enhance the anti diabetic activity of the compounds, Suter and Zutter (1965) have synthesised a series of substituted S-triazine derivatives.

Many heterocyclic hypoglycaemic agent similar in structure to biguanides were reported to have promising hypoglycaemic activity (El-Kerdawy *et al*, 1975). Pharmaceutical preparations containing heterocyclic guanidine compounds had been tested for their anti diabetic effect (Atso *et al*, 1982).

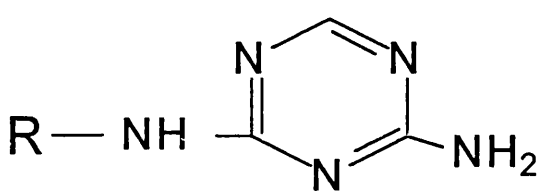


V



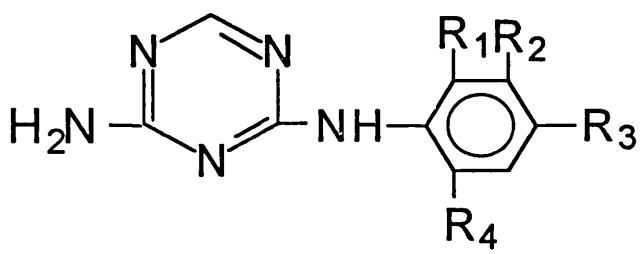
VI

One of the newer agents shown to reduce elevated glucose level in animal models are the guanamines (structure VII).



VII

These have been synthesised and evaluated as oral anti diabetic in rats (Gajewski *et al.*, 1983 a,b; Gajewski et al, 1984; Brzozowski *et al* 1980 a,b). Hypoglycaemic activity is found to be critically dependent on the structural characteristic of the group R. Certain of the variants of this series are the most active guanamine anti diabetic for example 2-amino-4-(5-ethyl-4 methyl-2-pyrazoline)-1,3,5-triazine. A comparison of these diamino-1,3,5-triazine derivatives with classical standards (phenformin and buformin) in several experiment show that these compound have hypoglycaemic activity e.g 6-amino-2-oxyl-1,2-dihydro-4 (3,5,5-trimethyl-2-pyrazolino)-1,3,5-triazines on oral administration to rats were comparable in hypoglycaemic activity to phenformin (Gajewski *et al* 1984). Monoacyl butyroguanamine also exhibits muscle relaxing and anti diabetic activity (Kreutzberger & Loch, 1985). Tamasdan and colleagues (1987) synthesised and tested seven new triazine derivatives including Urofort (structure VIII), with a view to establishing their effect on blood cholesterol, glucose, sodium and potassium concentrations, as well as on the gastric acidity in rats. All of the compounds induced moderate reductions in blood glucose, except Urofort.



VIII

Urofort = R1, R2, R3, R4 = H

The mechanism of action of the guanamines is, however unclear. This group, of compounds thus deserves further attention as a potential treatment of diabetes mellitus.

1.3. GLYCOGEN PHOSPHORYLASE

1.3.1. Historical Background

We can look back on more than 50 years of research on phosphorylase since Carl and Gerti Cori announced their discovery of the formation of glucose-1-phosphate from a reaction mixture containing glycogen , inorganic phosphate, and an aqueous extract of muscle containing phosphorylase and nucleotide adenylic acid (adenosine-5'- monophosphate, AMP), which activaties this enzyme (Cori & Cori, 1936). The reverse reaction, namely, synthesis of polysaccharides from glucose-1-phosphate by the action of phosphorylase was announced almost simultaneously in 1939 by Cori, Schmidt and Cori (Cori *et al.*, 1939).

For a number of years it was reasonably assumed that the *in vivo* function of phosphorylase was to catalyse both the synthesis and the breakdown of the 1,4-linkage of glycogen, until 1957 when Leloir and Cardini demonstrated that UDPG-glycogen synthase is the liver enzyme catalysing the direct transfer of glucose from uridine diphosphoglucose (UDPG) to glycogen. Further work led to the crystallisation of phosphorylase by Green and Cori (Green & Cori, 1943). A second form of the same enzyme was detected and crystallised later (Cori & Cori, 1945). Whereas the first form, the active or " a " form, is active without AMP, the second the " b " or inactive form requires AMP for activity. (Cori *et al.*, 1938). It nevertheless took 20 years for the development of the protein chemistry techniques through which Edwin Krebs and Edmund Fischer demonstrated in 1959, that phosphorylase a and b correspond to forms of the same protein in which a specific residue, Ser 14, is enzymatically phosphorylated or dephosphorylated, respectively. Thus, this simple chemical change is responsible for the profound differences in enzymatic properties of the two forms.

The current excitement stems from the work of Krebs, Fischer and Larner over the period 1955- 1970, when they discovered that neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the

phosphorylation state of glycogen phosphorylase (Krebs & Fischer,1956), phosphorylase kinase (Krebs *et al.*, 1959) and glycogen synthase (Friedman & Larner, 1963). The three enzymes remained the only examples of this phenomenon until the late 1960s, but the situation changed rapidly following the discovery of cyclic AMP-dependent protein kinase (Walsh et al., 1968).

In 1972 Graves and Wang observed that, while phosphorylase b is subject to control via the allosteric activation by AMP, it was inhibition by ATP, ADP, and glucose-6-phosphate. The discovery of cyclic AMP (cAMP) and the development of the concept of second messengers came from studies on the control of phosphorylase by glucagon and epinephrine which also led to the concept of the cascade of phosphorylation reactions. Shimazu and Amakawa (1975) were the first to show that glucagon was an activator of liver phosphorylase kinase. It was assumed, by analogy with muscle, that this was via a protein kinase and subsequent by using isolated rat hepatocytes, Vandenheede et al (1976) have provided evidence that phosphorylase kinase activation by glucagon is, indeed, caused by the action of cyclic AMP dependent protein kinase. In the late 1970s, it has become progressively apparent that cyclic AMP is not the sole messenger to signal changes in glycogenolysis and phosphorylase activation in the liver. Sherline *et al* (1972) were the first to show that the α-adrenergic agonist phenylephrine, produces an activation of glycogen phosphorylase in the absence of a rise in cyclic AMP in perfused rat liver. This discovery was not fully appreciated for several years possibly because it went against the widely prevalent view that cyclic AMP was the intercellular second messenger for glycogenolytically active hormones. The study of cyclic AMP- independent regulation of liver glycogenolysis received new impetus when it was shown (Hems & Whitton, 1973) that vasopressin induced an intense glycogenolysis in the perfused rat liver at concentrations likely to occur in haemorrhagic shock. Vasopressin is known to stimulate kidney adenylate cyclase but this activation of liver glycogen phosphorylase appeared not to be mediated by cyclic AMP i.e there was no increase in the liver content of cyclic nucleotide after the application of vasopressin (Kirk & Hems, 1974) and neither was liver protein

kinase activated in rats receiving an injection of vasopressin (Sudilovsky, 1974). It appeared therefore that vasopressin, as the α- adrenergic agents, acted in a cyclic AMP- independent way and required presumably, another intracellular mediator. Since cyclic AMP is not involved in the glycogenolytic effect of α- adrenergic stimuli, vasopressin or angiotensin, what could be the alternate second messenger? The most obvious candidate is Ca_i^{2+}, known to play a fundamental role in a whole series of cellular events and required for full catalytic activity of liver phosphorylase kinase (Shimazu & Amakawa, 1975, Khoo & Steinberg, 1975). The actions of Ca_i^{2+} can be mediated via calmodulin, (Cohen, 1980) which is known to be present as one of the four sub units of phosphorylase b kinase (Ramachandran *et al.*, 1985; Kee & Graves, 1986). Now it is well established that vasopressin, angiotensin II and α- adrenergic agonists stimulate hepatic glycogenolysis via a rise in cytosolic Ca^{2+} (Exton, 1983; Williamson *et al.*, 1985) by mobilising Ca_i^{2+} from the endoplasmic reticulum of the liver cell (Benedetti *et al.*, 1989; Somogyi & Stuki, 1991).

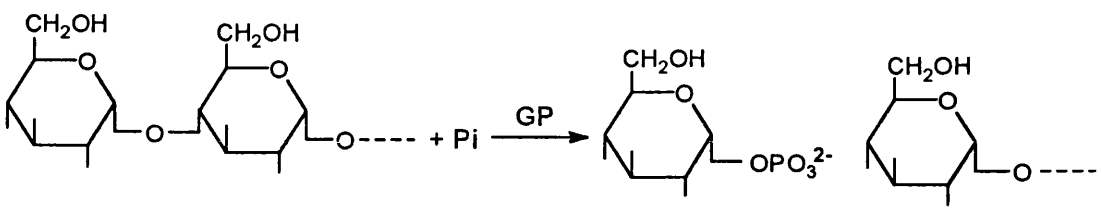
The activity of glycogen phosphorylase is allosterically controlled, as mentioned previously through AMP activation and ATP, G-6-P and glucose inhibition. Superimposed upon this allosteric control is control by enzymatic interconversion through the action of three enzymes:

1. Glycogen phosphorylase kinase, which specifically phosphorylates Ser 14 of glycogen phosphorylase b.
2. cAMP- dependent protein kinase, which phosphorylates and thereby activates phosphorylase kinase.
3. phosphoprotein phosphatase 1, which dephosphorylates and thereby deactivates both glycogen phosphorylase a and phosphorylase kinase.

We shall specifically focus on how the activity of glycogen phosphorylase is control by these three enzymes in detail in the next section.

1.3.2. Physiological Role and Regulation of Glycogen Phosphorylase.

Glycogen phosphorylase is the rate limiting enzyme for glycogenolysis (Cao, *et al* 1993). This enzyme catalyses the first step in the intracellular degradation of glycogen by adding inorganic phosphate across the α-1,4 - glucosidic links of glucose residues to form α-glucose-1-phosphate (Graves & Wang, 1972), the mechanism of glycogen phosphorylase is shown in Figure 1, phosphorylase removes glucose residues from glycogen by a phosphorolysis reaction. (Cao *et al* , 1993).



Glycogen (partial structure) α-D-Glucose 1-phosphate

Figure1. mechanism of glycogen phosphorylase

Glycogen phosphorylase is known to be highly regulated when catalyzing the breakdown of glycogen (Graves and Wang 1972,; Newgard *et al.*, 1989; Johnson, 1992). The mechanisms responsible for the regulation of glycogen phosphorylase are summarised in Figure 2. The enzyme is subject to allosteric activation by AMP and allosteric inhibition by glucose, ATP, ADP and glucose-6-phosphate (Newgard *et al.*, 1989). Although these effectors are considered to be of physiological significance in the regulation of glycogen metabolism, effector control of phosphorylase is integrated with a very elaborate control by covalent modification. phosphorylase exists in an " a " form, which is active, and a " b " form, which is inactive. These forms of the enzyme are interconverted by the actions of phosphorylase kinase and phosphoprotein phosphatase.

In response to nervous or hormonal signals , the enzyme is converted from the b form (predominantly T state) to the a form (predominantly R state) (Johnson *et al.*,

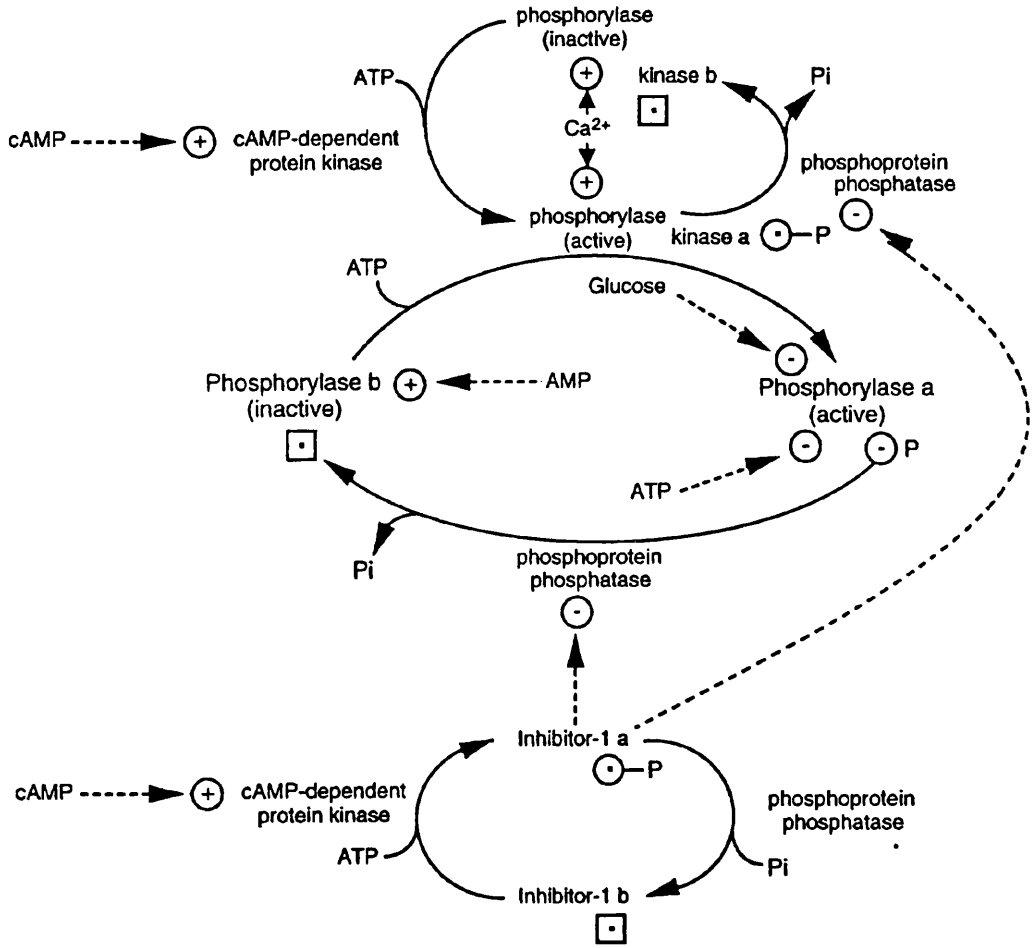


Figure 2. Regulation of glycogen phosphorylase by covalent modification.
(adapted from Pamela, C.C; Richard, A.H; 1994)

1989; Newgard *et al.*, 1989; Johnson, 1992) through the phosphorylase kinase catalysed addition of a phosphate to the hydroxyl group of Ser 14, located near the N-terminus of the molecule. Glucagon appears to increase the level of phosphorylase a by a concerted activation of phosphorylase kinase and inhibition of phosphorylase phosphatase (Stalmans *et al.*, 1990). There is also evidence that shows that glucagon and insulin have opposite effects on the activity of protein phosphatases in liver (Farkas *et al.*, 1986; Toth *et al.*, 1988; Bollen *et al.*, 1989). Phosphorylase kinase is responsible for the phosphorylation and activation of phosphorylase (Figure 2). Moreover, phosphorylase kinase itself is also subject to regulation by a cyclic phosphorylation - dephosphorylation mechanism. Cyclic AMP-dependent protein kinase is responsible for phosphorylation and activation of phosphorylase kinase (Cherrington *et al.*, 1976; Krebs & Beavo, 1979). Phosphoprotein phosphatase in turn is responsible for dephosphorylation and inactivation of phosphorylase kinase.

Phosphorylase kinase has been extensively purified from rat liver (DeWulf *et al.*, 1980). Its molecular weight is 1.3×10^6 daltons, similar to that of the muscle enzyme. It is a large enzyme, composed of four subunits with four molecules of each subunit in the complex ($\alpha 4 \beta 4 \gamma 4 \delta 4$). Catalytic activity resides with the γ subunit (Skuster *et al.*, 1980); $\alpha, \beta, \& \delta$ subunits exert regulatory control. The α & β subunits are phosphorylated in the transition from the inactive b form to the active a form of the enzyme. The α- and β- subunits are the components phosphorylated by cAMP-dependent protein kinase (Cohen & Antoniw 1973). Cyclic AMP- dependent protein kinase does not interact with phosphorylase directly, this protein kinase can only exert an effect on phosphorylase via its ability to phosphorylate phosphorylase kinase (Krebs, 1986). Thus, a bicyclic system is required for the activation of phosphorylase in respond to cAMP- mediated signals (Krebs, 1986).

The δ subunit of phosphorylase kinase also plays a regulatory role. The δ subunit is the Ca^{2+} - binding regulatory protein, calmodulin (Kee & Graves, 1986; Ramachandran *et al.*, 1985). Calmodulin is not unique to phosphorylase kinase but is often found in cells as the free molecule and is also bound to other enzyme complexes

(Sharma & Kalra 1994). Calmodulin functions as a Ca^{2+}-receptor in the cell, responding to changes in intracellular Ca_i^{2+} concentration and affecting the relative activities of a number of enzyme systems. The binding of Ca^{2+} to the calmodulin subunit of phosphorylase kinase changes the conformation of the complex, making the enzyme more active with respect to the phosphorylation of phosphorylase (Cohen, 1980). Note that in Figure 2, Ca^{2+} is shown as an activator of both phosphorylase kinase a and phosphorylase kinase b. This means that maximum activation of phosphorylase kinase require both the phosphorylation of specific serine residues of the enzyme and the interaction of Ca^{2+} with the calmodulin subunit of the enzyme. This is one mechanism by which Ca^{2+} functions as an important "second messenger" of hormone action.

It is obvious that activation of phosphorylase kinase by phosphorylation and Ca^{2+} will have a substantial effect on the activity of glycogen phosphorylase. It is equally obvious that turning off phosphoprotein phosphatase could achieve the same effect but ultimate control for the activation of phosphorylase would involve the simultaneous turning off of phosphoprotein phosphatase and turning on of phosphorylase kinase., and vice versa for the inactivation of the enzyme. Since phosphoprotein phosphatase also acts on phosphorylase kinase, turning off phosphoprotein phosphatase would also achieve greater activation of phosphorylase kinase.

Protein phosphatases are involved in liver glycogen metabolism at three levels : they assure the inactivation of phosphorylase and phosphorylase kinase, (Figure 2) and the activation of glycogen synthase (not shown). Little is known at present concerning the protein phosphatase that dephosphorylate the hepatic phosphorylase kinase. The protein phosphatase that acts on phosphorylase and glycogen synthase in the liver is classified as AMD (ATP, Mg^{2+}- dependent) or type-1, PCS (polycation- stimulated) or type- 2A, and possibly the Mg2+- dependent or type-2C (Cohen, 1989, Goris *et al.*, 1989). The AMD protein phosphatase (type-1) in liver and muscle contain the same (37kDa) catalytic subunit (Cohen *et al.*, 1989). Its appears always to be associated

with other polypeptides that determine its subcellular location (cytosolic, glycogen - bound, microsomal, nuclear) and its substrate specificity, and may govern other properties of indivdiual type-1 protein phosphatases (Bollen *et al.*, 1988; Schelling., 1988; Cohen, 1989). Type-1 protein phosphatase preferentially dephosphorylates the β -subunit of phosphorylase kinase (Cohen, 1989). The distinctive property of these phosphatases is their complete inhibition by two heat stable proteins: inhibitor-1, which becomes inhibitory upon phosphorylation by cAMP- dependent protein kinase (Figure 2) and inhibitor-2 (Cohen, 1989). The latter polypeptide has other regulator functions besides inhibitory properties and is therefore also referred to as the "modulator protein" (Merlevede *et al.*, 1984). Inhibition by modulator is frequently used as a tool to distinguish the AMD enzymes from the other protein phosphatases.

Inhibitor-1 has been identified in the liver of dog, pig, sheep, rabbit and guinea pig (Goris *et al.*, 1978; Macdougall *et al.*, 1989). In rabbit liver glucagon increases the extent of phosphorylation of inhibitor-1 (Macdougall et al., 1989) and this suggests that the direct actions of cAMP- dependent protein kinase could be reinforced by an inhibition of protein phosphatases (Stalmans *et al.*, 1990). However, inhibitor-1 does not qualify as a universal regulator since it cannot be detected in rat or mouse liver (Huang *et al.*, 1977; Macdougall *et al.*, 1989).

PCS protein phosphatases (type 2A) constitute a group of polymeric enzymes present in the soluble fraction of the cell (Waelkens *et al.*, 1987; Cohen, 1989). PCS protein phosphatases in liver contain two slightly different catalytic subunits. However, the different substrate specificities of specific PCS phosphatases have to be attributed to different regulatory subunits (Waelkens *et al.*, 1987). It can dephosphorylate the α subunit of phosphorylase kinase and is not affected by inhibitor proteins (Cohen, 1989).

Mg2+- dependent protein phosphatase is a cytosolic protein that was originally purified as a glycogen synthase phosphatase (Hiraga *et al.*, 1981).

The activation and inhibition of phosphorylase are under hormonal, neuronal and metabolic control. There are positive effectors that promote glycogen degradation such

as adrenaline or Ca_i^{2+} signals that results in activation of the enzyme by phosphorylation and direct activation, such as AMP and negative effector that inhibit degradation and allow the cell to store glycogen for later use, such as insulin-induced signals for enzyme dephosphorylation and glucose, ATP and glucose 6-phosphate which act directly on the enzyme.

1.3.3. The Effect of Diabetes on Hepatic Glycogen Phosphorylase.

An absolute or relative deficiency of insulin results in a disease called diabetes mellitus. Diabetes mellitus is a disease marked by excessive and uncontrolled blood glucose concentrations. High blood glucose is caused by lesser utilisation of the sugar by peripheral tissues, as well as overproduction by the liver in man. Several studies have demonstrated that glycogen phosphorylase is missing or reduced in diabetes and can be returend to normal by insulin. In this section we will focus our discussion on the effect of diabetes on glycogen phosphorylase.

The rate limiting step in hepatic glycogenolysis is catalysed by glycogen phosphorylase and this has been shown by several laboratories to be affected in the diabetic animals. In an earlier study, Hornbrook 1970 observed that alloxan diabetic rats had a lower concentration of glycogen and less phosphorylase activity than livers of normal rats. At the same year Gold (1970) demonstrated that, in diabetic animals, phosphoprotein phosphatase markedly declines and can be restored by insulin treatment. Khandelwal and colleagues (1977) in the study of the effect of streptozotocin - induced diabetes and insulin supplementation to diabetic rats on glycogen metabolising enzyme in liver, observed that the activity of the glycogenolytic enzymes were decreased in the diabetic animals. The enzyme activities were restored to control values by insulin therapy. The same observation was noted by Pugazhenthi & Khandelwal (1990) that diabetes was associated with the decrease of phosphorylase (a and total), cAMP- dependent protein kinase and phosphorylase kinase activities. However, phosphorylase phosphatase activity did not change .

Diabetes- related decreases in total phosphorylase activity have been observed using liver homogenates by Miller (1978a) who discovered that there were large decreases in hepatic glycogen associated with alloxan diabetes in fed rats and that these were accompanied by apparent decreases in total glycogen synthase, phosphorylase, protein kinase and synthase phosphatase activities. Bahnak and Gold (1982) examined the effects of diabetes on protein turnover in alloxan diabetic rats and concluded that rates of both synthesis and degradation of phosphorylase were increased in diabetic rat liver compared with control, they also observed a diabetes-related decrease in total phosphorylase activity and further concluded that increased degradation relative to synthesis must be responsible. In contrast Roesler and Khandelwal (1987) observed that the rate of phosphorylase degradation is decreased in the genetically diabetic mouse, while the synthesis rate remains unchanged, thus leading to the increased total phosphorylase activity in these animals.

Miller and colleagues have demonstrated that insulin together with T_3 (tri-iodothyronine) restores decreased glycogen synthase phosphatase in hepatocytes from diabetic animals (Miller *et al.*, 1986). Rulfs *et al* (1989) using primary cultured hepatocytes, showed that glycogen phosphorylase synthesis was significantly decreased compared with normal hepatocytes, suggesting that alterations in the rate of synthesis plays a major role in regulating the cellular phosphorylase content and consequently total activity.

The blunted stimulation of phosphorylase-a activity in the diabetic animals was influenced by a decrease in total cell activity of glycogen phosphorylase and decreased rise in cytosolic free calcium (increasing Ca_i^{2+} leads to stimulation of phosphorylase kinase and hence phosphorylase activation as discussed above) . Insulin replacement corrected both basal and stimulated changes in the acute diabetes model (Studer & Ganas, 1989).

Phosphorylase a is a potent inhibitor of glycogen synthase phosphatase, the protein phosphatase that activates glycogen synthase (Stalmans, 1976; Cohen., 1989). Protein phosphatase G is unique in that its synthase phosphatase activity is severely

inhibited by phosphorylase a (Mvumbi & Stalmans, 1987). This regulatory property has a key function in the activation of hepatic glycogen synthase in response to glucose. It is suggested that a separate subunit is responsible for the allosteric inhibition by phosphorylase a (Alemany & Cohen, 1986). The glycogen synthase phosphatase activity of hepatic protein phosphatase G almost disappears during the development of severe alloxan diabetes (Stalmans *et al.*, 1990). The defect accounts for the deficient activation of glycogen synthase in isolated hepatocytes from such rats (Stalmans *et al.*, 1987). The same defect occur in the liver of BB rats that develop a spontaneous insulin- dependent diabetes (Bollen *et al.*, 1988). In contrast, that glycogen synthase phosphatase activity is essentially normal in db/db mice with a non-insulin dependent diabetes. The phosphorylase phosphatase activity of protein phosphatase G is much less affected by a shortage of insulin (Stalmans *et al.*, 1987).

In studies on the effect of phosphorylases and kinases on hepatic glycogen metabolism during the fasted to refed transition in normal and streptozotocin- induced diabetic rats, it was shown that in diabetic livers phosphorylase (a and total) activities were significantly lower than those seen in the normal animals during the fasted to refed transition but the activity ratio in both group were comparable. cAMP-dependent protein kinase and phosphorylase kinase activity also showed substantial reduction in response to the meal. (Pugazhenthi & Khandelwal, 1991).

Recently it has been observed that mRNA levels for glycogen phosphorylase are affected by streptozotocin- induced diabetic rats (Barbera *et al.*, 1994). However the effect of diabetes on glycogen phosphorylase is not universal since it cannot be detected in some kinds of diabetic rats such as the non- insulin dependent diabetic Goto-Kakizaki rat (GK rats). (Zhang *et al.*, 1994).

The amounts of the four isoforms of catalytic subunits of type-1 protein phosphatase, PP1-alpha, PP1-gamma-1, PP1-gamma-2, and delta have been determined in extracts of various tissues in non- obese diabetic (NOD) mice. The amount of PP1- alpha were progressively decreased in livers of NOD mice as a

function of increasing concentrations of blood glucose, whereas the amount of PP1-gamma-1 and PP1-delta were unchanged (Takizawa *et al.*, 1994).

In summary it can be seen that diabetes markedly affects the enzymes of glycogen metabolism including glycogen phosphorylase and that the enzymes which regulate the

phosphorylase can be partially or completely restored to normal by treating the animals with insulin. The contribution of insulin to the regulation of hepatic glycogen metabolism has been demonstrated by many *in vivo* and *in vitro* studies which will be discussed in the next section.

1.3.4. The Effect of Insulin on Hepatic Glycogen Phosphorylase.

In the transition between feeding and periods of short fasting, the net hepatic glucose output is determined by the reciprocal activities of glycogen synthase (UDP glucose: glycogen 4-α- glucosyltransferase) and glycogen phosphorylase (1,4-α- D-glucan- orthophosphate α-glucosyltransferase). The regulation of the activation and inactivation of both of these enzymes is the object of various hormones, including insulin, glucagon and the catecholamines, and of glucose.

The first reported effect of insulin on glycogen phosphorylase was in 1967 when Bishop and Larner demonstrated insulin acting to decrease hepatic glucose output. It acts to promote glycogen synthesis by a direct activation of glycogen synthase but at the same time inactivates glycogen phosphorylase (Figure3).

It has become widely accepted that insulin promotes the arrest of glycogenolysis and the deposition of glycogen in the liver (Stalmans, 1976). The role of insulin in the regulation of hepatic glycogen phosphorylase has been the subject of *in vivo* and *in vitro* studies.

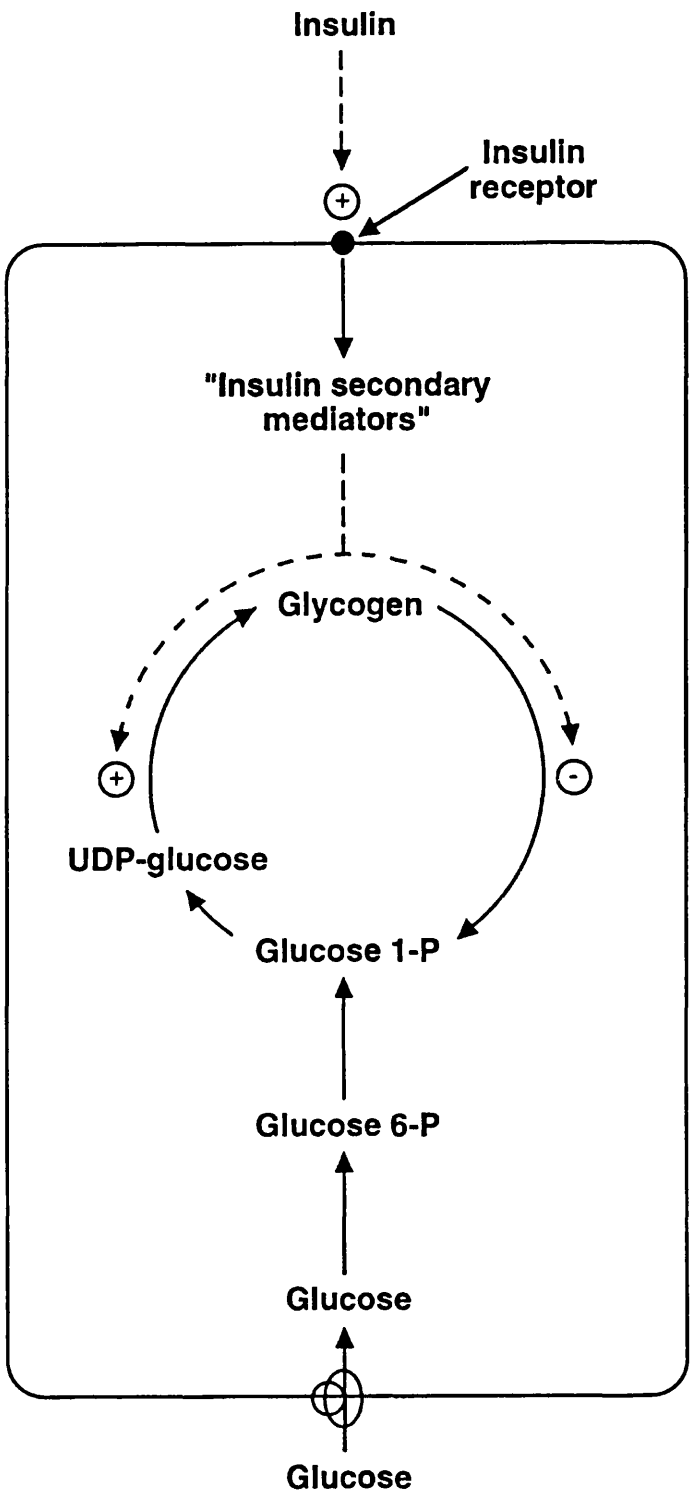


Figure 3. Insulin acts via secondary mediators of its action to inhibit glycogenlysis and promote glycogen synthesis in liver.

(adapted from Harris, R.A; 1992)

1. 3. 4. 1. *In vivo studies*

In vivo studies demonstrated a suppression of hepatic phosphorylase activity by insulin in the intact anaesthetised rat (Hers *et al.*, 1973). A partial inactivation of phosphorylase has also been observed in anaesthetised rats by Stalmans *et al* (1974) and also in the liver of the rhesus monkey with no significant change in hepatic cAMP concentrations (Curnow *et al,* 1975). Van derWerver and co-workers (1977) observed a similar inactivation in rabbit liver phosphorylase and phosphorylase kinase but not change in the hepatic concentration of cyclic AMP. The observation that insulin blocked the effect of hormones which elevated cAMP (Butcher, 1966) and reversed the processes which had already been stimulated in response to elevated cAMP (Robinson, *et al* 1971), led to the conclusion that insulin antagonism of these hormones is due to lowering of cAMP (Kono & Barham, 1973). However, Larner and co-workers (1979) proposed that insulin caused direct inhibition of cAMP- dependent protein kinase by inhibiting dissociation of its regulatory and catalytic subunits. In the effect of insulin on the enzymes regulating glycogen phosphorylase, it was demonstrated that insulin caused inactivation of cAMP dependent protein kinase was in skeletal muscle *in vivo* (Villear-Palasi & Wengen 1967). This was confirmed and extended by Shen *et al* (1970) and by Walaas et al (1972) in rat diaphragm and by Miller and Larner (1972, 1973) and by Gabbay and Lardy (1984, 1985, 1987) in adipocytes and hepatocytes and by Guinovart and co-workers (1987) in hepatocytes . Insulin - specific inhibition of the cAMP- dependent protein kinase was of great significance since it became the first bioassy for the putative insulin mediator.

Whereas the inactivation of phosphorylase is a constant feature after the administration of insulin, the activation of glycogen synthase occurs only occasionally. The sequential change in the activities of the two enzymes has been explained by the observation that glycogen phosphorylase a strongly inhibits glycogen synthase phosphatase (Hers, 1976; Stalmans, 1976). The authors suggested that the ability of insulin to activate hepatic glycogen synthase may be accounted for by this hormone's ability to inactivate phosphorylase protein phosphatase-1 (Alemany and Cohen, 1986).

Insulin stimulates dephosphorylation of both glycogen synthase (Roach, 1986) and phosphorylase (Zhange *et al.*, 1989) in skeletal muscle, thereby promoting enzymatic changes which favour glycogen accumulation. The same observation came from Dent that the reverse reaction of dephosphorylation that inactivates the enzyme is catalysed by protein phosphatase 1 (PP1), an enzyme that is regulated in response to insulin (Dent *et al.*, 1990). PP1 is the major enzyme that activates glycogen synthesis and also the principal enzyme that inactivates the enzymes of glycogenolysis (glycogen phosphorylase and phosphorylase kinase). In muscle, PP1 is regulated in response to insulin by phosphorylation of the glycogen binding subunit (Cohen, 1992). In the liver, the glycogen binding subunit does not appear to be controlled by phosphorylation / dephosphorylation events, and instead the dephosphorylation of glycogen synthase by hepatic PP1 is inhibited allosterically by low concentrations of phosphorylase a. This inhibition is mediated by the glycogen binding subunit (Alemany & Cohen, 1986; Bollen & Stalmans, 1992).

In hepatocytes, insulin has been shown to inhibit the stimulatory effects of phenylephrine on glycogenolysis that are considered to be mediated through a rise of cytoplasmic Ca^{2+} (Blackmore *et al.*, 1979). This observation was confirmed by observations from Somogyi which demonstrated that insulin, the physiological counterpart to the gluconeogenic agonists, suppresses phenylepherine- induced Cai^{2+} oscillations (Somogyi *et al.*, 1992).

1. 3. 4. 2. *In vitro studies*

In vitro studies clearly demonstrated that in hepatocyte cultures in serum free medium, when insulin and pro insulin are added as the sole hormones, a decrease in the activity of glycogen phosphorylase occurs (Beyner and Geelen, 1981; Hartmann *et. al,* 1987). Insulin reduces the glucagon - stimulated elevation of cAMP and activation of phosphorylase (Exton *et al.*, 1971; Gabbay & Lardy., 1984). The glucagon-stimulated [^{14}C]-glucose release was decreased by insulin and pro insulin (Hartmann *et al.*, 1987).

The same observation was also reported by Deeg *et al* (1993) and this was associated with an appropriate reduction in phosphorylase activity, confirming the role of this enzyme as the primary regulatory site for glucose release from glycogen (Revers, 1984; Probst *et al.*, 1985; Hartmann *et al.*, 1987).

The direct effect of insulin on either intracellular levels of cAMP or cAMP-dependent protein kinase activation of phosphorylase in hepatocytes (Corbin *et al.*, 1985) and inhibition of lipolysis in adipocytes (Beebe *et al.*, 1984) in response to various cAMP analogs, both in the absence and presence of insulin have been studied. It was observed that insulin blocked the effects of only those analogues which were readily hydrolysed by the low Km PDE (phosphodiesterase). In both these systems, there was a good coordination between the inhibitory effects of insulin and the apparent Km of cAMP analogs for PDE, thus suggesting that insulin activates a low Km PDE both in adipocytes and hepatocytes (Beebe et al., 1984). Similar studies to those described above for adipocytes and hepatocytes were also done with isolated cardiomyocytes (Corbin *et al.*,1986; Corbin *et al.*, 1988) and concluded that, in contrast to adipocytes and hepatocytes, insulin regulates glycogen phosphorylase through a mechanism which is independent of PDE activation or the other components of the cAMP cascade (Sheorain, 1990).

An inositol phosphoglycan (the polar head group of a glycosyl phosphatidylinositol) has been considered as a putative mediator of insulin action. Fetal and adult hepatocytes were incubated with insulin or inositol phosphoglycan after which glycogen phosphorylase activity was determined. Inositol phosphoglycan mimicked the action of insulin on both forms of the enzyme from adult hepatocytes with regard to the inhibition of glycogen phosphorylase (Ruiz et. al, 1993).

When insulin was added to cultures of rat hepatocytes before arginine vasotocin (which increased tissue cAMP concentration and thus activate glycogen phosphorylase), it inhibited the actions of arginine vasotocin, which indicated that insulin activates cAMP phosphodiesterase and so reduces the concentration of cAMP (Janssens & Grigg, 1993).

The mechanism by which insulin directly inhibits glycogen phosphorylase still remains unclear. Recent evidence strongly suggests that an intermediate step involves activation of protein phosphatase activity. Thus insulin has been shown to stimulate protein phosphatase 1 activity in rabbit skeletal muscle (Dent *et al.*, 1990), 3T3L1 cells (Chan *et al.*, 1988), 3T3D1 cells (Villa-Moruzzi, 1989), rat epitrochleris muscle (Zhang *et al.*, 1989), as well as in rat fibroblasts tranfected with the human insulin receptor cDNA (Begum *et al.*, 1993).

Recently it was found that insulin blocked the effect of amylin in increasing the phosphorylation of phosphorylase in rat diaphragms (Lawrence & Zhang, 1994). As noted above, the significance of this data is that glycogen phosphorylase is insulin sensitive enzyme activity and can, thus, be used to measure insulin- like activity.

1.3.5 The Role of Glucose in Regulating Glycogen Phosphorylase Activity in Hepatocytes.

1. 3. 5. 1. In the normal animal

Glucose has been recognised to be an important regulator of hepatic glucose output. This observation extends from the initial studies of Soskin *et al* (1938) who noted that the level of glycemia controls, in part, net hepatic glucose uptake or output. In *in vivo* studies it was observed that when glucose was administered, there was a conversion of phosphorylase from the phospho- to dephospho- form (Stalmans & Hers *et al.*, 1973). Stalmans *et al* (1974) demonstrated that glucose first caused the inactivation of phosphorylase in the liver and then an activation of glycogen synthase. In *in vitro* studies, addition of glucose to the incubation medium reproduces these changes in the isolated perfused liver and in isolated hepatocytes (Hue *et al.*, 1975). vanderWerve *et al* (1977) demonstrated that when hepatocytes were incubated in the presence of high concentrations of glucose, glycogen phosphorylase was strongly inactivated with no change in the activity of phosphorylase kinase. The current

explanation is that glucose binds to phosphorylase a and that the resulting complex is a superior substrate for phosphorylase phosphatase, hence the inactivation of phosphorylase and the inhibition of glycogenolysis (see Figure 4). Glucose- induced inactivation of glycogen phosphorylase, is explained by the enhanced exposure of the phosphorylase- glucose complex to the action of protein phosphatase (Stalmans,1976).

The removal of phosphorylase a, which is a strong inhibitor of glycogen synthase phosphatase, allows the latter enzyme to convert synthase b into the a form, which in turn determines the rate of glycogen synthesis (Stalmans, 1976; Curnow and Larner, 1979; Hers, 1981). There is, however, a difference between these observations and those of Carabaza et al (1992). The latter authors observed, when they examined the compounds; 2-deoxyglucose, 5-thioglucose, 6-deoxyglucose, 1,5-anhydroglucitol and 3-O-methylglucose in the same fashion as glucose, that glucose is the molecule that triggers the inactivation of phosphorylase but that this inactivation is not the signal for glycogen synthase activation. They also demonstrated that glucose 6-phosphate was the signal for glucose synthase activation. Ciudad et al (1986) have shown that, in rat hepatocytes, the intracellular concentration of glucose-6-phosphate shows a positive correlation with the activation of glycogen synthase, suggesting that glucose - 6- phosphate rather than glucose could constitute the signal for glycogen synthase activation.

Glucose inactivates glycogen phosphorylase a by competitive inhibition with the substrate glucose-1-phosphate and by favouring the T-state conformation of the enzyme. The T-state conformation is a better substrate for the protein phosphatase than the R-state (Sprang et al., 1982, Maden et al., 1983) and thus increases the formation of glycogen phosphorylase b, relieves the inhibition of PP1 and promotes the activation of glycogen synthase. Thus, the balance between glycogen degradation and glycogen synthesis is controlled by phosphorylation and dephosphorylation events, and these events are modulated by other metabolites, notably glucose.

Since glucose is a physiological regulator of hepatic glycogen metabolism state (Witters & Avruch, 1978; Hartmann *et al.*, 1987). and weak inhibitors of glycogen

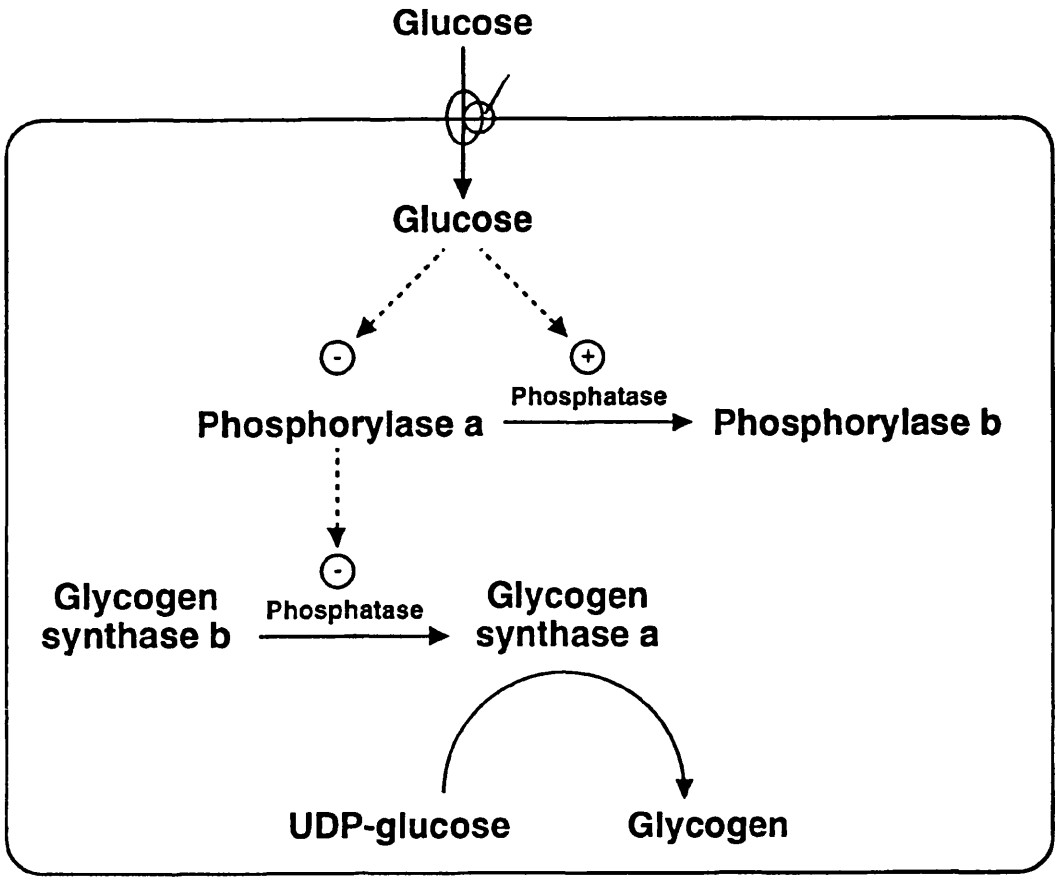


Figure 4 Overview of the mechanism responsible for glucose inhibition of glycogenolysis and stimulation of glycogen synthesis in the liver.

(adapted from Harris, R.A; 1992)

phosphorylase are weakly hypoglycaemic (Kasinsky *et al.*, 1978), Martin *et al* (1991) investigated a number of glucose analogue inhibitors of glycogen phosphorylase as potential drugs for treating diabetes. Several α and β anhydroglucohetonic acid derivatives and 1-deoxy-1- thio- β- D- glucose analogues have been synthesised and tested in a series of crystallographic and kinetic binding studies with glycogen phosphorylase. It has been postulated that, in the liver, glucose analogues with greater inhibition of glycogen phosphorylase may be more effective regulatory agents than glucose. Recently Watson and co-worker (1994) reported crystallographic and kinetic investigations of a number of α- and β glucoheptonamides and β- thioglucose analogue inhibitors of T-state rabbit muscle glycogen phosphorylase based on guidelines previously established (Acharya *et al.*, 1991; Martin *et al.*, 1991).

The idea that inhibitors of glycogen phosphorylase are useful hypoglycaemic agents is, thus, not novel but an establised line of enquiry.

1. 3. 5. 2. In the diabetic animal

The first reported effect of diabetes on hepatic glycogen metabolism was made in 1967 when Kreutner and Goldberg demonstrated that an increased glucose load activated hepatic glycogen synthase in normal rats but was ineffective in alloxan diabetic rats. Results from Miller and co-worker (1973) confirmed the direct effect of glucose to activate glycogen synthase in normal perfused liver and in addition demonstrated that glucose had no effect on hepatic synthase in perfused liver from alloxan diabetic rats. The lack of effect of glucose on glycogen synthase in diabetic liver was also associated with a lack of inactivation of glycogen phosphorylase and no increase in net incorporation of [^{14}C]-glucose into glycogen. Although the studies demonstrated an impairment in synthase activation when liver from diabetic rats were perfused with 10mM glucose for 15 min, it was not determined whether synthase activation could occur with higher glucose concentrations or longer perfusion times.

Miller (1977) extended his studies by using different concentrations of glucose (10-30mM) and perfusion times (5-90 min) and observed that whereas glucose infusion into livers from normal rats resulted in activation of glycogen synthase and inactivation of glycogen phosphorylase, glucose was completely ineffective as a modulator of synthase and phosphorylase in perfused liver from diabetic rats regardless of perfusion time or glucose concentration. Whitton and Hems (1975) reported a defect in glucose-induced glycogen synthesis in perfused livers of starved rats made diabetic with streptozotocin. These observations plus those of Miller (1977) that the defects in glucose modulation of synthase and phosphorylase in liver of diabetic rats can be corrected by treating the animals with insulin or glucose plus fructose, suggested that the enzymatic apparatus for glycogen synthesis was not totally lacking in diabetic rat liver. Ciudad *et. al.* (1988) also demonstrated that, with fructose and fructose plus glucose, the enzymatic system for glycogen synthesis can be operative in diabetic rat liver. Interestingly, they also demonstrated that inactivation of phosphorylase by glucose is not impaired in diabetic hepatocytes. although basal phosphorylase a activity is lower than in normal hepatocytes. Further evidence that glucose- induced inactivation of glycogen phosphorylase worked normally in diabetic rats was provided by Bollen *et. al.* (1983) when they studied the effects of glucose on phosphorylase and glycogen synthase in hepatocytes isolated from acutely (40h) and chronically (90h) alloxan diabetic rats. The latter study did not substantiate the hypothesis of Miller (1979), discussed above, but proposed that glucose did induce some inactivation of phosphorylase, and speculated that the degree of diabetes could be a possible explanation. The reason for this lack of agreement may be that in earlier work changes in phosphorylase activity were obscured by an assay that was not specific for the a form of phosphorylase.

There is, thus, evidence to suggest differences in response of glycogen phosphorylase to insulin and / or glucose between normal and diabetic rats. It is, therefore, necessary to investigate the effect of any potential insulin-mimetic drugs in both normal and diabetic animals.

1.4. ASSAY OF GLYCOGEN PHOSPHORYLASE.

The cell disruption buffer was used at pH 6.1 (rat liver phosphorylase b is less active at low pH) and contained NaF which is a necessary addition in the assay to prevent the conversion of phosphorylase a to b and as an inhibitor of phosphorylase phosphatase. To the assay mixture caffeine is added in order to render the assay specific for phosphorylase a and inhibit phosphorylase b. Under these conditions, the activity of phosphorylase b is about 1% of that of phosphorylase a (Stalmans & Hers, 1975).

1.5. STEROID METABOLISM.

Endogenous and exogenous steroids are metabolised by complex enzyme systems which are both cytochrome P450- dependent and - independent. The cytochrome P450-dependent monooxygenases are probably the most important oxidase system being responsible for the oxidation of endogenous substrates such as fatty acids, steroids, vitamins and also for the biotransformation of xenobiotics that gain entry into the living organism. It achieves this broad specificity by existing as a number of structurally distinct families of proteins, each with a characteristic with a substrate specificity (Lu & West, 1980; Gonzalez, 1990). Numerous stereospecific steroid hydroxylations serve as sensitive fingerprints for characterising individual forms of cytochrome P450 (Arlotto *et al.*, 1991); e.g testosterone 6β-, 2α-, 7α- and 15 α- hydroxylase and androstendione 16β- hydroxylase are, respectively, specific indicators of rat cytochrome P450 3A, 2C11, 2A1, 2A2, and 2B1 expression (Waxman, 1991 a,b; Arlotto *et al.*, 1991; Pampori & Shapiro, 1994). Identifying individual monohydroxylated products of commonly used P450 substrates, such as testosterone and androstenedione, is however complicated by the fact that each steroid

can be metabolised to more than 20 hydroxylated derivatives (Waxman, 1991 a; Arlotto *et al.*, 1991).

In this study, we employed the well-characterised steroid substrate, androst- 4-ene- 3,17- dione, and the metabolic routes of this steroid are illustrated in Figure 5. 4-androstene- 3,17-dione is a convenient and easy substrate to use, being readily available in both an unlabelled and (^{14}C)- labelled form. Metabolism of this steroid substrate has been well characterised by Gustafsson and Stenberg (1974) and the metabolites can be readily separated in the laboratory by one- dimensional thin layer chromatography. Some instruments like high- performance liquid chromatography (HPLC) and gas chromatography mass spectrometry (GC-MS) are often used for the identification of steroid metabolites by comparison to authentic standards (Waxman & Walsh , 1983; Waxman, 1991 a,b; Arlotto *et al.*, 1991; Pampori & Shapiro, 1994). These methods are, however, expensive, time-consuming, and inappropriate for dealing with a large number of samples simultaneously. In contrast, silica gel thin-layer chromatography (TLC) is inexpensive and has advantages of rapidity of analysis combined with simplicity of the set-up needed for processing a large number of samples. Several laboratories have identified P450-dependent hydroxylases using TLC.

Cytochrome P450- dependent monooxygenases hydroxylate 4-androstene-3,17-dione predominantly at the 7α-, 6β- and 16α- positions. The male specific cytochrome P4502C11 has been shown to catalyse 90% of the metabolism of 4-androstene- 3,17-dione at the 16α- position (Waxman, 1984) and the same isoenzyme also hydroxlates testosterone at the 16α- position (Morgan *et al.*, 1985). 4-androstene-3,17- dione hydroxylation at the 6β- position is thought to be catalysed by cytochrome P450 3A2 (Waxman *et al.*, 1985), although direct assessment of the activity of the purified enzyme has not yet been determined. Steroid hormone 6β- hydroxylation is also catalysed by several other family 3A P450s, including rat CYPs 3A1 and 3A2 (Waxman *et al.*, 1985; Nagata *et al.*, 1990). Cytochrome P4502A1 is responsible for 7 α- hydroxylation of 4-androstent- 3,17-dione (Waxman *et al.*, 1988) and it therefore

appears in the uninduced liver, 4-androstene-3,17- dione metabolism is thus catalysed predominantly by three separate and selective cytochrome P450 isoenzymes.

5α- reductase is another 4- androstene- 3,17- dione metabolising enzyme but, although it is a membrane -bound microsomal enzyme, it does not contain cytochrome P450 as an integral component. It has been shown, however, that 5α- reductase exists as part of a multicomponent system which possesses many similarities to the cytochrome P450 monooxygenase system. The electron transport chain is composed of three enzymes; steroid 5α- reductase, coenzyme Q10 and NADPH- cytochrome oxidoreductase. In this system, coenzyme Q10 is a cytochrome and electrons are passed from NADPH to coenzyme Q10 by NADPH- cytochrome oxidoreductase and thence from coenzyme Q10 to the steroid substrate by 5α- reductase, where they participated in the reduction of the double bond (Figure 5) (Golf & Graf, 1978). The final enzymes involved in the metabolism of 4-androsten-3,17-dione are 17α/β and 3α/β hydroxysteroid dehydrogenase, both of which are microsomal, although details of their mode of action remain to be fully elucidated.

1.5.1. The Effect of Diabetes Mellitus and Insulin On Androst-4-Ene 3,17-Dione Metabolism.

Diabetes mellitus is known to affect drug and steroid metabolism in the rat liver. In rats, it has been clearly demonstrated that chemically induced diabetes mellitus produce changes in hepatic microsomal drug metabolism (Reinke *et al.*, 1978). Overall testosterone metabolism was significantly increased in streptozotocin diabetic liver microsomes, whereas it was markedly decreased in alloxan diabetes. Mixed function oxidase activity towards aminopyrine was similar affected. Glucuronidation reaction rates towards morphine, oetsrone and 4-nitrophenol were also markedly distinct in both models as well as after insulin treatment. This demonstrates that diabetogenic agents modify the isoenzymes of cytochrome P-450 differently and selectively reduce the synthesis of certain UDP-glucuronyltransferase forms (Vega *et al.*, 1993).

Reports from various studies have indicated that streptozotocin (STZ)-induced diabetes mellitus can influence hepatic steroid metabolism in the rat (Leaming *et al.*, 1982; Subbiah & Yunker, 1984). The effect of diabetes on hepatic steroid metabolism can be prevented or reversed by insulin administration to diabetic animals. An early report by Skett (1986), that STZ - induced diabetes mellitus exerts a substrate and sex -dependent effect on hepatic steroid metabolism in the rat which could be reversed by insulin treatment, indicated that insulin plays a role in the regulation of steroid metabolism.

The diabetic state resulted in an overall decrease in the rate of microsomal metabolism of androst- 4- ene- 3,17- dione, compared to control. A differential effect on metabolism was observed with individual metabolites. The hepatic microsomes from the diabetic rats exhibited an increase in the female- specific 7α- hydroxylase (Stenberg, 1976) and decreases in the male- specific 16α- hydroxylase, indicating a sex - differentiation in the effect of the diabetic state. All of these alteration were restored to normal when the diabetic rats were treated with insulin. This results is in good agreement with previous reported work relating to drug metabolism where the diabetic state produced a sex- dependent effect by interfering with androgen- dependent microsomal pathways in the rat (Kato & Gillette, 1965; Kato *et* al., 1971). Skett (1986) also reported the sex- dependent effect of diabetes mellitus on steroid metabolism in rat where alterations were only seen in the male and the effect was always to abolish the sex differences in steroid metabolism found in the intact animals. In the late 1980s work was concentrated on the alteration in cytochrome P-450 isoenzyme complement in the diabetic state. Cytochrome P-450 RLM5 (P-4502C11) has been shown to be the major from of cytochrome P-450 responsible for 16α-hydroxylase activity (Waxman, 1984). Favreau and Schenkman (1987) showed that testosterone 16α- hydroxylase activity was decreased in diabetes and this was accompanied by a dramatic decrease in immunodetectable cytochrome P-450 RLM5. Insulin treatment resulted in partial restoration of the 16α- hydroxylase activity and the cytochrome P-450 RLM5 levels.

Further evidence of alterations in specific constitutive cytochrome P-450 isoenzymes in the diabetic state was provided by Schenkman (1990) when he found that in diabetes there is a change in the microsomal P450 composition, with form 2C11 (cytochrome P-450 RLM5) declining and 2A1 increasing (cytochrome P-450 PCN2) which is responsible for 7α- hydroxylation of 4-androstene -3,17- dione. One might also expect to see a fall in levels of form 3A2, which normally is responsible for about 95% of microsomal testosterone 6β- hydroxylase activity and this form does actually decline (Thummel & Schenkman, 1990).

Shimojo *et al* (1993) studied changes in cytochrome P450 isozymes in both hepatic and renal microsomes of rats with diabetes caused by streptozotocin and they demonstrated that the amounts of cytochrome P450 2A1 and 3A2 increased in hepatic microsomes of diabetic rats and P450 2CII decreased. Treatment with insulin restored these to the levels in the control. The catalytic activities of testosterone 7α- and 6β-hydrxylase were high in the hepatic microsomes of diabetic rats and testosterone 16α-hydroxylation activities were low. These changes were reversed by insulin treatment. The induction and suppression of cytochrome P450 isozymes in diabetic rat were consistent with the changes in the catalytic activities.

Phenformin and tolbutamide have been shown to have a direct effect on the liver in elevating androst-4-ene 3,17-dione metabolism. They are able to mimic the effect of insulin in normal and diabetic rat hepatocytes with respect to androst -4-ene 3,17-dione metabolism. In normal rat hepatocytes phenformin has been demonstrated to cause a dose-dependent increase in all the enzyme activities. In diabetic hepatocytes phenformin still exhibited a dose-dependent increase in all the enzyme activities. In the same study using normal rat hepatocytes, insulin in the presence of phenformin selectively produced greater effect on the 6β- hydroxylase, 17-OHSD and 5α-reductase activities than with insulin or phenformin alone. It seems that phenformin may inhibit the ability of insulin to increase the activity of 7α- and 16α-hydroxylases. In the diabetic hepatocytes, phenformin shows no potentiation of the effect of insulin on any of the enzymes except the 7α and 6β hydroxylase activity at physiological

insulin concentrations. In normal rat hepatocytes, tolbutamide elicited a dose-dependent increase in androst-4-ene-3,17-dione metabolism similar to that exhibited by phenformin. The mechanism by which tolbutamide increased all the steroid enzyme activities is unclear. In the diabetic rat, tolbutamide was still able to increase all the enzyme activities. In the normal rat, tolbutamide selectively inhibits the insulin-stimulated 5α-reductase activity. With regard to the other enzymes, increasing insulin concentrations potentiated the effect of tolbutamide when compared to tolbutamide alone. Similar to phenformin, tolbutamide reduces the responsiveness of insulin-stimulated steroid enzyme activities but the mechanism responsible for this is yet to be determined (Hussin, 1988).

The insulin - mimetic effects of vanadate in preventing the increase in the level and activity of several P450 proteins in streptozotocin- diabetic rats has also been examined. The diabetic state caused by the pancreatic beta cell toxin streptozotocin results, like the diabetes of genetic origin, in major alterations in expression of the P-450 isozymes (Verrecchia & Guaitani, 1993).

1.5.2. The Effect of Insulin on Androst 4-ene 3,17 dione Metabolism in Normal Male Rat Hepatocytes.

The role of insulin in rat hepatic drug and steroid metabolism is known indirectly from the effect of diabetes mellitus on both the above liver parameters since insulin is the principal hormone altered in the diabetic state.

In vivo parenchymal liver cells however, are exposed to many different hormonal stimuli and this makes it difficult to assign effects seen to particular hormones. For instance when animals are made diabetic by chemical treatment, growth hormone and glucagon serum levels are also affected and the effects seen on microsomal metabolism could be due to an effect of these hormones. The use of isolated liver cells would be expected to be an ideal solution to this problem (Hussin & Skett, 1987; Liddle *et al.*, 1992).

Hussin (1988) observed that insulin increased all of the enzyme activities at physiological concentrations of the hormone in isolated rat hepatocytes. This is in contrast to the selective effect of insulin reported by Skett (1986) in the intact rat. In relation to the effect of insulin on steroid metabolism, in hepatocytes, the overall inhibition of the cyclic AMP-dependent protein kinase and the adenylate cyclase activity (Gabbay & lardy, 1987; Saltiel, 1987; Villalba *et al.*, 1988) and stimulation of the cyclic AMP - dependent phosphodiesterase by this inositol phosphate-glycan (Kiechle & Jarett, 1980; Saltiel *et al.*, 1986) would theoretically decrease the phosphorylation of cytochrome P450 to its inactive cytochrome P420 (Pyerin *et al.*, 1987) therefore, would explain the increased amount of substrate (i.e. androst-4-ene-3,17-dione) being metabolized.

PHASE 1 METABOLISM OF ANDROST-4-ENE-3,17-DIONE

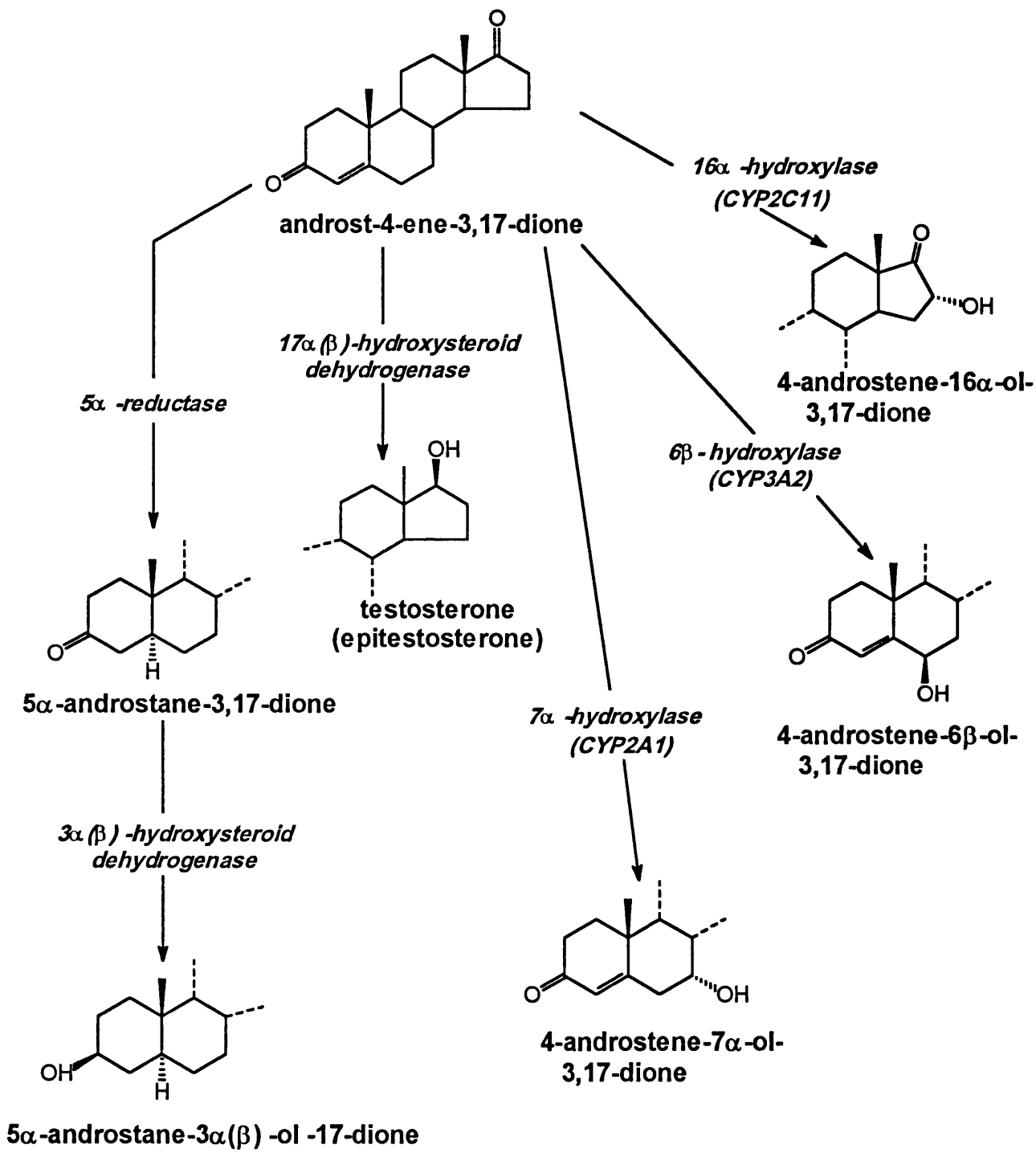


Figure 5. The phase 1 metabolism of androst -4-ene- 3,17-dione in the liver. Specific isoenzymes thought to produce the various metabolites are given in the capital italic

1.6. LIVER CELL PREPARATION

The liver occupies a central position in body metabolism. Its size, softness, and relative homogeneity have made it a favourite organ for biochemical investigation. For the study of intact liver functions under controlled conditions the isolated, perfused rat liver has been extensively used. This experimental system is excellent for many purposes, but has several major shortcomings: (1) the liver as an organ is not completely homogeneous, containing up to 40% nonparenchymal cells (Daoust, 1958): (2) it is difficult to obtain many identical samples from one liver, and impossible to test different experimental treatments simultaneously; and (3) the viability of an isolated liver can be maintained only for a limited period of time (8-10 hours). Numerous attempts have been made to overcome these problems by the isolation and purification of intact parenchymal liver cells. The early mechanical and chemical methods for liver cell preparation were relatively successful in converting liver tissue to a suspension of isolated cells, but unfortunately nearly all such cells were damaged. Isolated hepatocytes are now the most widely employed experimental model to investigate hepatic function (1987; Gabbay & Lardy, 1987)

1.6.1 Methods

The cell preparation methods used by most laboratories concerned with hepatocyte culture have been based on the two-step collagenase perfusion procedure of Seglen (1976), although variations of the one-step procedure have also been used successfully (Flaim *et al*; 1985). Almost every laboratory has its own modification of these methods and undoubtedly most of these give satisfactory preparations. In general, the aims should be to obtain the maximum yield of hepatocytes whilst minimising biochemical changes in the cells during isolation, minimizing contamination with non-hepatocytes and maximising the proportion of cells capable of attaching and

surviving in culture. In the final preparation this must be carried out under sterile conditions. There are only a few publication which discuss different collagenase perfusion isolation procedures in relation to the subsequent behaviour of cells in culture (Williams *et al.*, 1977; Kreamer *et al.*, 1986).

1.6.2. The Role of Collagenase.

The introduction of collagenase as a liver- dispersing enzyme by Howard *et al.*, (1967) greatly facilitated the preparation of intact cells, and when Berry and Friend (1969) introduced the use of physiological liver perfusion to make the tissue uniformly accessible to the action of collagenase, it became possible to prepare intact liver cells in high yield and convert the whole liver to a suspension of intact cells, i.e. both the initial yield and the cellular viability approach 100% under ideal circumstances (Seglen, 1972). The collagenase perfusion method is vastly superior to all previous techniques, and is likely to remain the ultimate method for the preparation of rat liver cell.

1.6.3. The Role of Ca^{2+}.

Ca^{2+} has been found to play a dual role in liver dispersion. The inclusion of Ca^{2+} during perfusion with collagenase enhances enzymatic activity and accelerates dispersion. For the enzymatic dispersion to be effective, Ca^{2+} must be removed from the tissue by a pre perfusion, and then added again upon perfusion with collagenase (Seglen, 1972). The removal of Ca^{2+} can be accomplished by perfusion with EGTA or EDTA (Seglen, 1972) or, provided the perfusion is effective, simply by pre perfusion with a Ca^{2+} free buffer, thus combining the washout of blood and Ca^{2+} (Seglen, 1973). Since the effect of Ca^{2+} removal is irreversible, it must be due to some conformational change in the intercellular matrix which favours dispersion. Modjanova and Malenkov (1973) have described a Ca^{2+}-dependent hepatic adhesion factor which

is washed out of the mouse liver upon perfusion with a Ca^{2+}-free medium. It would therefore seem likely that the dispersion -promoting effect of preliminary Ca^{2+} removal is due to the detachment and washout of this adhesion factor, in particular since the divalent catin specificity is the same in both cases.

1.6.4. The Use of a Two- Step Procedure.

Since the introduction of the two-step procedure for collagenase perfusion, several workers have adopted the practice of including Ca^{2+} along with collagenase (Berg & Boman, 1973; Barnabei *et al.*, 1974; Baur *et al.*, 1975; Christoffersen & Berg, 1974; Edwards & Elliott, 1974; Nilsson *et al.*, 1974; von Bahr *et al.*, 1974; Williams & Gunn, 1974). Cells isolated by the two-step technique have been reported to retain K^+ better, and to have a higher K^+ uptake capacity, than cells prepared without Ca^{2+} addition (Barnabei *et al.*, 1974); this has also been shown with cells prepared from liver slices incubated with collagenase in the presence of Ca^{2+} (Howard *et al.*, 1973). Cells prepared with Ca^{2+} activated collagenase furthermore have, on the average, a higher membrane potential than cells prepared without Ca^{2+} (Baur *et al.*, 1975).

The two-step approach was introduced by Seglen (1976) as a consequence of his studies of the effects of collagenase on isolated perfused liver (Seglen, 1972). It was observed that the liver swelled more rapidly on addition of collagenase and 5mM Ca^{2+}, than when collagenase alone was added, provided that there had been a preliminary perfusion with a medium when was very low in Ca^{2+}. Seglen argued that since collagenase activity is dependent on Ca^{2+}, and since a virtual absence of Ca^{2+} is a prerequisite for cell separation, it was good practice to first perfuse the liver with a medium free of Ca^{2+} and subsequently perfuse with a Ca^{2+} -rich medium containing collagenase.

1.6.5. The Level of Glycogen.

Levels of glycogen depend very much on the nutritional state of the donor animal. The level is highest in fed animals and is very low in the cells of rats fasted for 18 h . The levels in isolated cells correspond to those in the intact organ. The highest cell content of glycogen is observed in cells obtained from meal-fed animals (Katz et. al; 1975). However, to retain cell glycogen during the preparative procedures, it is necessary to ensure a level of at least 15mM glucose in the perfusion and washing media. If, in the subsequent experimental study, the cells are to be incubated in the high concentration glucose, then- glucose must be added at the last washing solution. Under these circumstances, it is particularly important to ensure that a temperature close to 0-4°C is maintained during the washing procedures or else considerable glycogen breakdown will occur at this stage. The standard temperature for incubation of rat hepatocytes is 37°C. Temperature only a few degrees higher will rapidly cause cell damage (Bowers *et al.*, 1981).

1.6.6. The Incubation Time with Test Drug.

The observation from our laboratory on the incubation time of the biguanides, guanamines and insulin on the activity of glycogen phosphorylase suggests that the time course of activation and inactivation is very rapid, occurring within minutes if not seconds. The administration of insulin to rabbits caused a rapid inactivation of phosphorylase which, within 3 minutes, lost about 60% of its activity. Phosphorylase kinase was concomitantly inactivated. In contrast, insulin did not change the hepatic concentration of cyclic AMP (vanderWerve *et. al,* 1977). Van derWerve and co-workers also demonstrated that the addition of glucagon to a suspension of isolated

hepatocytes activated phosphorylase kinase and phosphorylase. This effect was at its maximum within 2 minutes of the addition of the hormone and then decreased progressively during the following 30 minutes. During the same experiments, the dose-dependence of glucagon, was measured 2 minutes after the addition of the hormone and its antagonism by insulin.

A phospho-oligosaccharide which is the polar head group of a novel insulin-sensitive glycophospholipid has been implicated in insulin action. Alvarez and coworkers (1987) have investigated the insulin-like effects of this phospho-oligosaccharide on glycogen phosphorylase a and pyruvate kinase activities in hepatocytes incubated in the presence of glucagon. Both insulin and phospho-oligosaccharide antagonise the action of glucagon after 5 minutes to 75% of original glycogen phosphorylase activity. Glucose caused an important inactivation of liver phosphorylase within 1-2 minutes (Stalmans *et al.*, 1974). Further studies of isolated rat hepatocytes have show that this lag is the time required for the inactivation of glycogen phosphorylase by different concentrations of glucose with or without K+ ions in the external medium (Hue *et al.*, 1975; Witter & Avruch, 1978). 2-Deoxyglucose and 5-thioglucose, in the same fashion as glucose, caused the inactivation of rat hepatocyte glycogen phosphorylase. The change was preceded by a short lag (2-5 minutes). In hepatocytes isolated from fed rats, sulfonylureas (tolbutamide and glipizide) in the incubation medium caused significant activation of glycogen phosphorylase. The greatest effects were observed two minutes after sulfonylurea addition (Mojena *et al.*, 1989; Alarcon *et al.*, 1993). Phenacyl imidazolium compounds have been shown previously to lower blood glucose levels without causing lactic acidemia in fed dogs, fed rats, and obese- diabetic insulin resistant mice and rats but not in starved or insulin- dependent streptozoctocin diabetic rats (Dominianni & Yen, 1986). In studies conducted with isolated hepatocytes, phenacylimidazolium activation of glycogen synthase and inactivation of glycogen phosphorylase, the time course was in the order of 5 min (Harris et al., 1989). The glucagon stimulated activation of glycogen phosphorylase in rat hepatocytes occured within 4 min (Deeg, et. al, 1993).

The literature thus, agrees with our proposed timing of 5 minutes as the pre incubation time for effects on glycogen phosphorylase.

1.7. AIMS OF THE PROJECT.

1. To study the effect of synthesized biguanides and guanamines on the activity of glycogen phosphorylase in normal rat hepatocytes as a measure of their insulin - like action. The biguanides such as phenyl-, phenylethyl-, benzyl were cyclized into triazines to see the effect of cyclization on the activity of the compounds and to determine the effect of changing substituents in various positions.
2. To determine the structure - activity relationship of compounds synthesized for their insulin-like action.
3. To investigate the effect of guanamines (cyclised biguanides) on the activity of glycogen phosphorylase in cultured hepatocytes from STZ diabetic rats.
4. To see the effect of guanamines (cyclised biguanides) on the activity of glycogen phosphorylase in the cells isolated from insulin treated diabetic rats.
5. To determine the effect of guanamines (cyclised biguanides) on the activity of glycogen phosphorylase from normal rats in the presence of different concentrations of glucose.
6. To determine the effect of guanamines (cyclised biguanides) on the activity of glycogen phosphorylase in hepatocytes from STZ diabetic rats in different glucose concentration.
7. To determine the effect of guanamines (cyclised biguanides) on androst -4- ene-3,17- dione metabolism in normal rat hepatocytes.

The overall aim was to see if guanamines had any direct insulin-like actions on hepatocytes which would suggest that they may be useful.

MATERIALS AND METHODS

MATERIALS AND METHODS

The methods can be separated into three main sections

(1) The synthesis of benzylbiguanide, N phenylguanamine (1), N phenethylguanamine (2) and N benzylguanamine (3).

(2) The assay of the effect of test compounds on glycogen phosphorylase a in isolated rat hepatocytes.

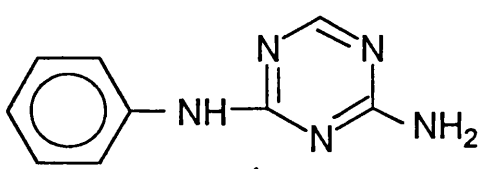
(3) The assay of the effect of test compounds on the metabolism of androst 4-ene-3,17-dione in isolated rat hepatocytes.

The methods used in each of these three sections is summarised below.

2.1 CHEMISTRY

2.1.1 Synthesis of Benzylbiguanide and Guanamines

The work to be discussed in this report is concerned with the synthetic pathways to some triazines, to be tested as potential antidiabetics, namely N phenylguanamine (1), N phenethylguanamine (2) and N benzylguanamine (3).



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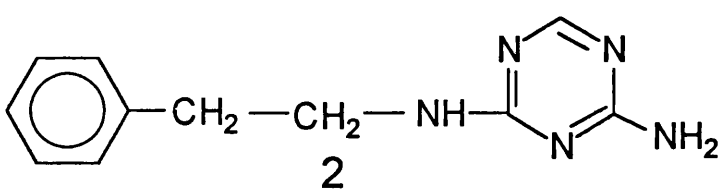

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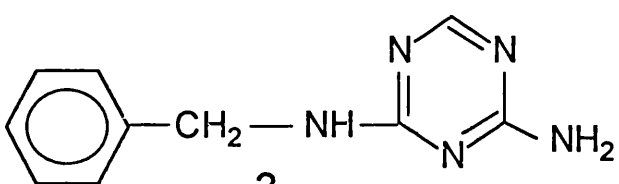

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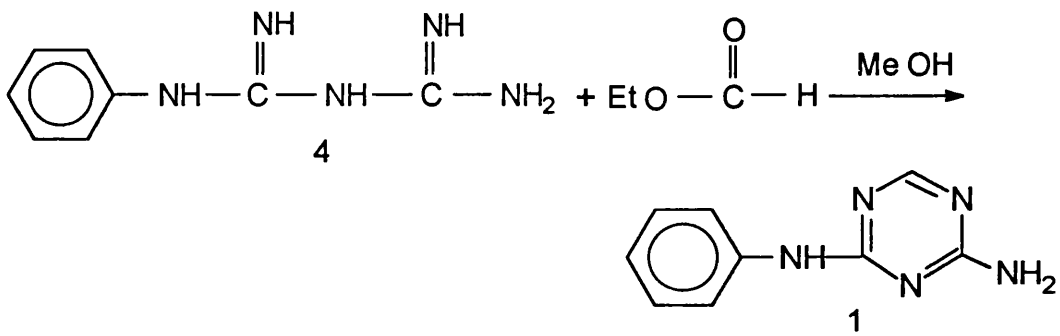
Phenylguanamine (1)

Phenylguanamine (1) was synthesized by the method of Overberger *et al* (1954). Phenylbiguanide (4) (Aldrich Chemical Company Inc.) was dissolved in methanol and ethyl formate added to give the product (1) in 69% yield. Microanalysis (C, H and N) of the product gave values in agreement with theory. The melting point agreed with the literature value (see Overberger *et al* 1954). The synthetic method is shown in Scheme I.



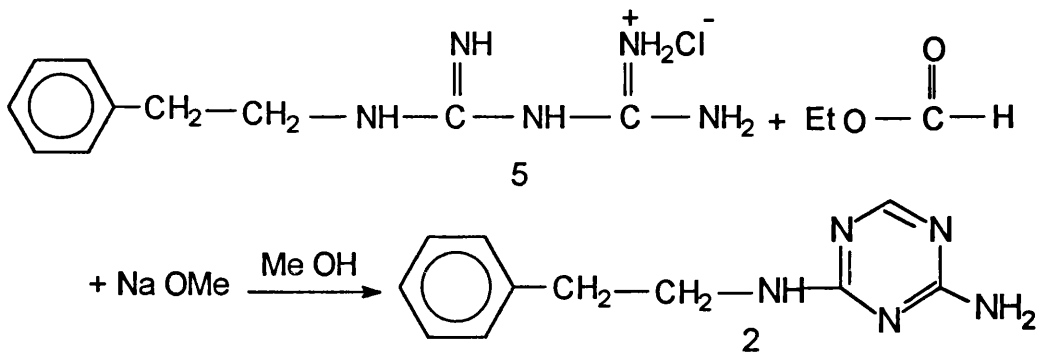
Scheme I

Phenylguanamine(1)hydrochloride.

Phenylguanamine hydrochloride was prepared from phenylguanamine (1) by addition of methanol:conc hydrochloric acid (5:1) to pH 2.5, according to published procedures (Shapiro *et al* 1957).The solvents were removed by distillation and the residue was dissolved in water .Addition of sodium chloride with stirring precipitated the hydrochloride of phenylguanamine.

Phenethylguanamine (2)

Phenethylguanamine (2) was prepared from phenethylbiguanide hydrochloride (5) (Sigma Chemical Co.) and ethyl formate in the presence of sodium methoxide as shown in Scheme II.



Scheme II

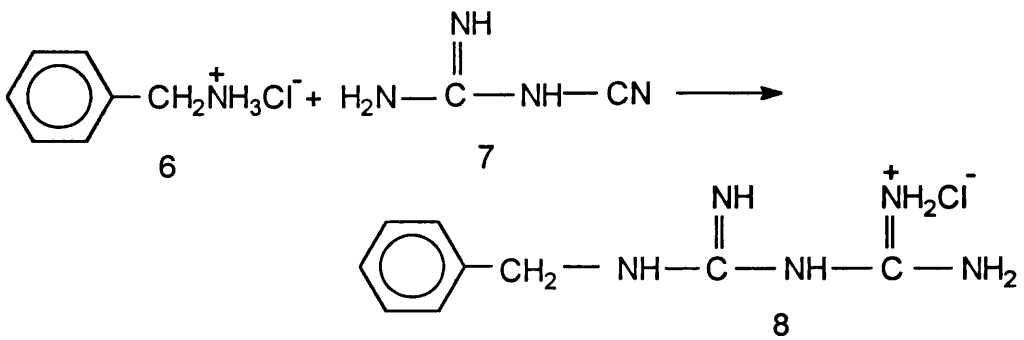
Phenethylguanamine (2) hydrochloride

Phenethylguanamine hydrochloride was prepared from phenethylguanamine (2) by addition of methanol : concentrated hydrochloric acid (1:1) to pH 2.5, according to the published procedure (Shapiro *et al* 1957). The solvents were removed by distillation and the residue was dissolved in water. Addition of sodium chloride without stirring precipitated the hydrochloride of the phenethylguanamine (2). The melting point was in agreement with the literature value (Shapiro *et al* 1957), also the microanalytical data agreed with theoretical values.

Benzylbiguanide hydrochloride (8)

Benzylamine hydrochloride was prepared from benzylamine and methanolic hydrogen chloride as described in the experimental section.

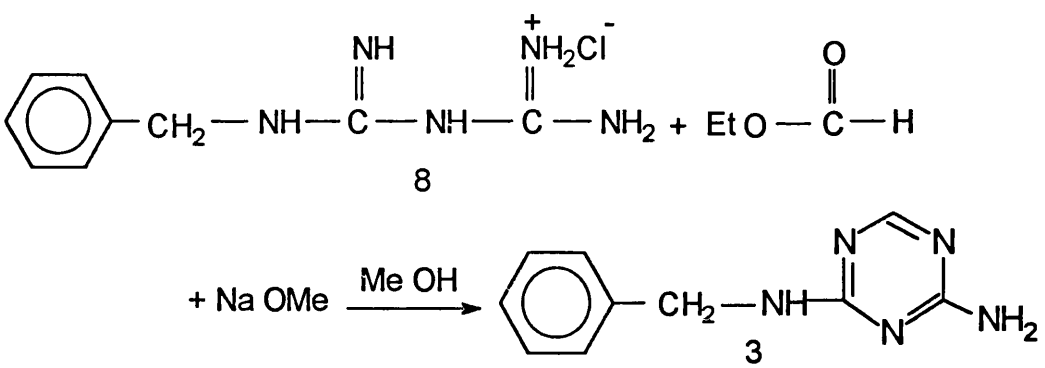
Benzylbiguanide hydrochloride (8) was prepared (Erienmyer et al 1899) (Scheme III) by melting benzylamine hydrochloride(6) and dicyandiamide(7) together.



Scheme III

Benzyl guanamine(3)hydrochloride.

Benzyl guanamine (3) hydrochloride was prepared (Shapiro et al 1957) by treating the benzylbiguanide (8) with methanol containing sodium methoxide and methyl formate was added. The product was converted to the hydrochloride as described in the experimental section. The synthetic method is shown in Scheme IV



Scheme IV

2.1.2 Experimental Section

1H NMR spectra were recorded on either a Perkin Elmer 90 MHz or a Bruker 200 MHz spectrometer. Spectra were recorded with d6DMSO [(CD3)2SO] as the solvent and Me4Si as the internal standared. 1H and 13C NMR spectra were recorded by Dr. D. Rocroft and Mr. J. Gall with a Bruker AM 200 Sy and WP 200 Sy spectrometer. Combustion analysis for C, H and N were performed by Mr. Kim Wilson using a Carlo Erba Analyser, Mod. 1106. Melting points were taken on a Kofler hot stage apparatus.

2-Amino-4-Phenylamino-1,3,5-Triazine (Phenylguanamine) (1)

Phenylbiguanide (4) (Aldrich Chemical Company) (1.77 g, 0.01 mol) was dissolved in methanol (7.5 ml) and ethyl formate (0.74 g, 0.01 mol) was added at room temperature. The product began to precipitate in 5 min and a thick slurry was formed within 1 h. The solid was filtered off after being left standing overnight and was washed with water to give phenylguanamine (1) (1.31 g, 72%). The product crystalised from dioxane as small white needles, m.p. 235-236°C (lit.m.p. 235-236 °C). The product Rf 0.82 was shown to be pure by TLC on silica plates developed with ethanol, water, formic acid (5 :1 :1). Analytical TLC plates were viewed under an ultra violet lamp (254 nm) or were developed by iodine vapour. Phenylguanamine (1) (Found C, 57.7; H, 4.7; N, 37.45 Calc for C9H9N5: C,57.7; H,4.8; N,37.4%) _H(,200 MHz) 6.98 (t, J. 7.3 Hz, P-phenyl-H), 7.02 (brs,NH2,exch with D2O), 7.28 (2H,t,J 8.3Hz. m. phenyl-H), 7.77 (2H, d. J .7.6 Hz O-phenyl-H), 8.20 (S, 6-H) and 9.51 (S, Ph NH, exch with D2O),_c (50.3 MHz) 119.9 (O-Ph-C), 122.0 (P. Ph. C) 128 (m-ph-c) and 139.6 (ipso-Ph-C), 163.7 (C-2 or -4) 165.9 (C-6) and 166.2 (C-4 or -2).

2 - Amino - 4 - Phenylamino -1, 3, 5- Triazine (phenylguanamine) (1) hydrochloride

Phenylguanamine (2.98g) was converted into the hydrochloride by dissolving in methanol : hydrochloride (6:1) to pH 2.5. The solvents were removed by distillation and the residue (2.6397g) dissolved in 39.5ml of water. Addition of sodium chloride (7.9g) with stirring precipitated the hydrochloride of phenylguanamine.This was filtered off and washed with saturated sodium chloride. The solid was dried at 70°C in a dryer pistol under vacuum, recrystallized from isopropyl alcohol by dissoliving in a minimum of solvent (20), with heating under reflux. It was kept at room temperature overnight, filtered off, washed with isopropyl alcohol and, dried at 70°C under vacuum. Yield (1.36g,60.8%), m.p. 200-201°C, (Found:C, 48.20; H, 4.48; N,31.39; Cl, 15.83 Calc for C9H10N5Cl; C, 48.32; H, 4.47; N, 31.32; Cl, 15.88). _H (200 MHz) 6 (brs, NH2, exch with D2O), 7.15 (t. J7.3Hz, p-phenyl-H), 7.35 (2h, t.J7.8Hz. m.phenyl-H), 7.7 (2H, d.J.7.7Hz. o-phenyl-H), 8.45 (s.phNH, exch with D2O), 8.55 (s.6-H) and 8.9 (brs,NH,exch.with.D2O), _C (50.3MHz), 122 (p.ph.c), 125 (o-ph-c), 129 (m,ipso-ph-c,c-6), 137.4 (c-2or-4), 157.5 (c-4or-2)

2- Amino- 4 - Phenethylamino - 1,3,5 - Triazine (Phenethylguanamine) (2)

Phenethylbiguanide hydrochloride (5) (2.42g, 0.01mol) (Sigma Chemical Co.) was dissolved in methanol (10.3 ml). Ethyl formate (0.74g, 0.01mol) and sodium methoxide (0.54g, 0.01mol) were added at room temperature. The product began to precipitate immediately and a thick slurry was formed. The solid was filtered off after being left standing overnight and was washed with water to give phenethylguanamine (2) (1.32g, 61.6%). Phenethylguanamine (2) crystallised from dioxane as rectangular plates, m. p. 159-160°C (Lit. m.p. 159-161°C). (Found: C, 61.13; H, 6.07; N, 32.36 Calc for C11H13N5: C, 61.39; H, 6.05; N, 32.56%). _H (200 MHz) 2.81 (2H, t, J 7.9Hz, CH2), 3.48 (2H, t, J 7.9Hz, CH2), 6.71 (br S, NH2, exch with D2O), 6.85 (br

S, Ph NH, exch with D2O) 7.26 (5H, m. ph) and 7.92 (S, 6-H); _c (50.3 MHz) 34.7 CH2, 41.5 CH2, 126, 128, 129 and 139.5 (ph), 164.5 (C-4), 165 (C-6) and 166 (C-2).

2-Amino-4-Phenethylamino-1,3,Triazine(Phenethylguanamine)(2) Hydrochloride

A mixture of (6.32g, 0.04mole) phenethylamine hydrochloride and (3.36g, 0.04mol) of dicyandiamide was ground until it became fine powder then it was heated and stirred in an oil-bath maintained at 148-150°C. The reaction mixture, upon reaching 130°C, was completely fluid and was maintained with continued stirring for 1hr. The reaction mixture was then mixed with a solution of 30.6ml of methanol containing (2.2g, 0.94mol) of sodium methoxide, (1.7g, 0.03mol) of methyl formate was added and the reaction mixture was allowed to stand for 24hr. The mixture was treated with 1:1 methanol-hydrochloric acid (conc) 26ml at pH 2.5. The solvents were removed by distillation under vacuum (Rota vapour), the residue (7.8g) was dissolved in water (18.3ml). Addition of aqueous sodium chloride (20g/100ml) to the reaction mixture with stirring quickly precipitated the hydrochloride of phenylethylguanamine. The mixture was kept overnight at room temperture then it was filtered off and washed with saturated sodium chloride. The solid was dried at 70°C in a vacuum and recrystallised from isopropyl alcohol by dissolving in the solvent (11.4ml) with heating under reflux. It was kept at room temperture overnight, filtered off and then washed with isopropyl alcohol, dried at 70°C under vacuum. Yield (4.24g, 42%), phenethyl guanamine hydrochloride recrystallized from isopropyl alcohol as, plates, m.p 185-186°C (Lit 185-186°C, Shapiro *et al* 1957). (Found: C, 52.24; H, 5.69; N, 27.92; Cl, 14.16 Calc for C11H14N5Cl; C,52.48; H,5.56; N; 27.84; Cl, 14.11) _H (200 MHz) 2.89 (2H, t, J7.4Hz, phCH2), 3.56 (2H, t, J 7.5Hz, NHCH2.exch with D2O), 7.3(5H, m, ph), 8.1 (brs, NH2 exch with D2O) 8.4 (S, 6-H), 9.18 (1H, t, J10.35, NH exch with D20), _C (50.3MHz), 34 (C-4), 42 (C-4), 126.5, 129.5, 130, 130 and 139 (ph), 156 (C-6), 157 (C-4) 160 (C2).

Benzylamine Hydrochloride

Methanol (4.5ml) and conc hydrochloric acid (4.5ml) were added gradually to benzylamine (5.35g, 0.05mol) until the pH was 2.5. The mixture was kept overnight and the methanol was then removed by vacuum distillation. The residue of the benzylamine hydrochloride (6.39g) was dried in a drying pistol at 40°C under vacuum for 2h. This salt had a m.p of 257-259°C (Lit. 255-257°C) it was seen under the microscope to be crystalline.

Benzylbiguanide Hydrochloride(6)

A mixture of (5.7g, 0.04mol) of benzylamine hydrochloride and (3.4g, 0.04mol) of dicyandiamide was ground until it became powder then it was heated and stirred in an oil-bath at 148°-150°C. The reaction mixture upon, reaching 130°C, was completely fluid, then it quickly solidified to light yellow crystals, it was removed and cooled. 3ml of hot water was added and left to stand overnight. The product was filtered off and washed with 3ml of water then it was recrystalize in 3ml of water under reflux for 10 min . The product was removed by filtration after standing overnight and and dried at 70°C under vacuum. Yield (3.97g, 43.5%), m.p 197°-198° (lit m.p 197°C). (Found: C, 47.36; H, 6.42; N, 30.61; Cl, 15.66 Calc for C9H14N5Cl; C, 47.26; H, 6.56; N, 30.63; Cl, 15.53). _H (200 MHz) 4.4 (2H, d, J 5.97Hz; CH2NH exch with D2O) 7.05 (s, NH, NH, 2NH2 exch with D2O), 7.35 (5H, m.ph), 7.98 (1H, t, J 5.97Hz, NH exch with D2O), _C (50.3 MHz), 44 (c-4), 127, 127.5, 127.5, 129, 129, 139 (ph), 158.5 (C2), 160.5 (c4)

Benzyl Guanamine (3)Hydrochloride

A mixture of (5.7g, 0.04mole) of benzylamine hydrochloride and (3.4g, 0.04mole) of dicyandiamide was ground until it became powder then it was heated and stirred in an oil-bath at 148°-150°C. The reaction mixture upon reaching 130°C, was completely fluid and immediately solidified as a light yellow gummy solid when it was removed and cooled. The cooled reaction mixture was then treated with a solution of 30ml of methanol containing (2.2g, 0.04mole) of sodium methoxide and (1.7g, 0.03mole) of methyl formate was added .The mixture was treated with 1:1 methanol-hydrochloric acid to pH2.5 (19.5ml). The solvents were removed by distillation and the residue (7.2g) dissolved in 120ml water. Addition of sodium chloride (20g/100ml of reaction mixture) precipitated the hydrochloride. The product was filtered off, washed with saturated sodium chloride, dried and recrystalized from isopropyl alcohol. Dried at 70°C under vacuum. Yield (4.24g, 44.6%), m.p 200°-201°C (lit m.p 200°-202°C) (Found: C, 50.15; H,5.25; N, 29.20; Cl, 14.81 Calc for C11H14N5Cl: C, 50.31; H, 5.45; N, 29.35; Cl, 14.88), _H (200 MHz) 4.55 (2H, d, J 6.18Hz CH2NH exch with D20), 7.25 (5H.m.ph), 8.1 (brs, NH2 exch with D2O), 8.4 (S, 6-H), 9.5 (1H, t, J. 16.7Hz, NH exch with D2O), _C (50.3 MHz), 43.5 (C-4), 3 (127.5), 2 (128.5), 137 (ph), 165 (C-6), 158 (C-4), 161 (C-2).

2.2. PHARMACOLOGICAL METHODS

2.2.1 EXPERIMENTAL ANIMALS

Mature male rats of the Wistar strain, bred in the Department, were used throughout the study. The animals were allowed free access to water and food (CRM Nuts, Special Diets Services, Essex) and housed in light and temperature controlled condition (19±1°C, lights on 7:00-19.00) and were used when they were 10-12 weeks old at the begining of the experiment. When comparing groups, age-matched animals were used and each group consisted of at least seven animals. The weights of individual rats ranged from 250-300g and the variation within each group was ±25g.
In experiments where diabetes mellitus was induced by streptozotocin (STZ), the rats were weighed before treatment and then 7-10 days post-treatment prior to sacrifice.

2.2.1.1 Induction of Diabetes by Streptozotocin

Diabetes mellitus could be induced in experimental animals by administering to the animals streptozotocin (STZ) . STZ is transported into pancreatic β-cells through glucose transporters in the cell membranes and attacks mitochondria. Mitochondrial ATP generation is inhibited and the resulting high concentration of intacellular ADP causes its degradation providing hypoxanthine, a substrate of xanthine oxidase (XOD) whose activity is intrinsically very high in the β-cell. The, XOD-catalyzed reaction produces uric acid and oxygen radicals, but β-cells are inefficient in scavenging these radicals because of their extremely low activity of superoxide dismutase. On the other hand, STZ directly activates XOD and enhances oxygen radical generation. Consequently, the pancreatic β-cells are subjected to a high concentration of oxygen radicals when exposed to STZ (Kawad, 1992).

The intravenous injection of a single high dose of STZ exerts direct toxicity on the β-cell, which results in necrosis within 48-72h and overt permanent hyperglycaemia (Wang et al, 1993). Since STZ is unstable in solution , it was dissolved in distilled

water immediately before use and then injected intravenously (60mg/kg) into the tail vein of the rat under halothane/nitrous oxide anaesthesia. The tail was rubbed to expose the vein before injecting the drug. Control animals were treated identically except that they were injected with vehicle only. The animals were left for 7-10 days in conventional animal cages and the weight of the animal was taken regularly during this period. The diabetic state was assessed by loss of weight (15%) and measurement of blood glucose as described below.

2.2.1.2. Insulin Treatment of STZ-Diabetic Rats

Insulin (from porcine pancreas, 24 I.U per mg) replacement, 4-8 I.U./100g body weight, subcutaneously was begun 48-72h after STZ injection as described above, a time when the diabetic state was well established. Daily determination of blood glucose was performed by the glucose oxidase method as described below and the dose of insulin adjusted as necessary to maintain blood glucose levels at or near to normal.

2.2.2 ISOLATION OF HEPATOCYTES

Hepatocytes were isolated by a modification of the two-step collagenase perfusion technique of Seglen (1976). The solutions were saturated for about 10 minutes with a mixture of oxygen and carbon dioxide (95:5 v/v) before the perfusion to meet the oxygen demands of the metabolically active parenchymal cells. This is important, since the avoidance of hypoxia has been shown to be necessary in the preparation of hormone-sensitive cells (Zahlten & Stratman, 1974). Other precautions taken were good temperature control and the use of the shortest exposure of the liver to collagenase (Berry *et al*, 1991). All buffers used in the isolation procedure had been previously heated in a water-bath set at 37°C. The temperature of the perfusate reaching the liver was maintained at 37°C for the duration of the perfusion process by

storing the buffer in an insulated reservoir set at 45°C before pumping it into the liver. It should also be noted that any air bubbles in the system were eliminated using a simple bubble trap. While the rat was under anaesthesia (4% halothane; 800ml/min oxgyen : 800ml/min nitrous oxide using a Mini Boyle Anaesthetic Machine), (Figure 6) the abdomen was cut open and the portal vein was freed of any fat. 1000U of heparin was injected via the tail vein as an anticoagulant prior to commencing the perfusion.

A cannula (size 20 GA x 32mm length) was inserted into the hepatic portal vein at a position just before it branches into the liver and held in place with a piece of surgical string (1001/40). The needle was removed from the cannula to allow blood to flow back up the cannula. the inferior vena cava was cut and the tube from the pump was attached to the cannula. The thoracic cavity, via the diaphragm, was opened and the superior vena cava cut. Perfusion (using a Watson Marlow 501U perfusion pump) was commenced with 500ml of Ca^{2+}-free Krebs-Henseleit buffer (see Composition of Buffers below) at the rate of 35-40ml/min. The Ca^{2+}-free conditions are necessary for the disruption of the tight junctions, which intimately connect the parenchymal cells and also the absence of Ca^{2+} during the first perfusion is essential for the effectiveness of the subsequent enzymatic dispersion (Seglen, 1972). In later experiments, 0.1mM EGTA was incorporated into the first perfusion buffer to enhance the Ca^{2+}-extracting properties of the perfusion. The dispersion was even more effective when EGTA was used in the first perfusion due to its calcium chelating properties. If EGTA was used in the first buffer, this was flushed out with 80ml Ca^{2+}-free Kreb's-Henseleit buffer without EGTA as EGTA inhibits the action of collagenase used in the subsequent perfusion. This perfusion was followed by a recirculating perfusion with 100ml Krebs-Henseleit solution containing 0.3 IU/mg of collagenase and 4.2mM $CaCl_2$. The buffer was allowed to run through for 1min to wash the first buffer out of the system and then the collagenase buffer was recycled for 9-11min (or until the liver looked as if it is falling apart and gentle pressure on the lobes appeared to release the cells). By this method of collagenase perfusion it is possible to convert the whole liver to a suspension of intact cells. After the perfusion, the liver was removed and placed in

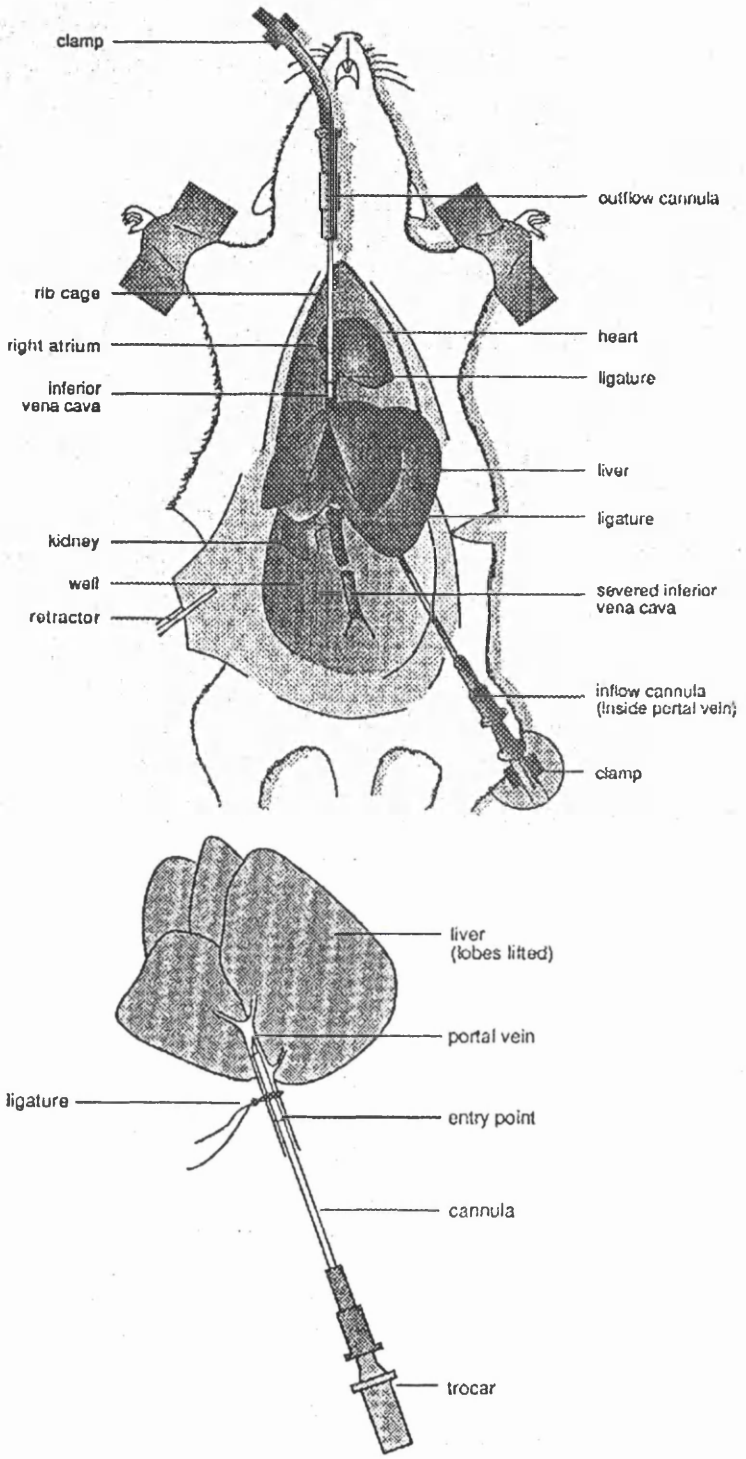


Figure 6. Isolation of hepatocytes and placement of portal cannula

warm washing buffer. The Glisson's capsule was gently removed and the liver disrupted by gentle scraping with a spatula. The suspension of cells was filtered through gauze (Snowflake absorbent gauze) to separate connective tissue and undigested liver. Parenchymal cells were then harvested after washing with washing buffer (see Buffers below) and resuspending 3 times. The cells were sedimented between each wash by centrifugation at 200g for 2 min at a temperature of +5°C in a Damon-IEC refrigerated centrifuge. The supernatant containing non-parenchymal cells and damaged parenchymal cells was discarded each time. The cells were kept on ice for one hour to equilibrate.

The number of cell was counted in a haemocytometer. In the case of parenchymal liver cells, the intact cells with their well-defined outline and refractile appearance are readily distinguished from the flattened, ground-glass-looking damaged cells even without the addition of a vital stain such as trypan blue (Howard et al 1967). 10μl of cell sample was taken and a 1 in 100 dilution carried out using washing buffer. Each large square of the haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1cm^3 is equivalent to approximately 1ml, the subsequent cell concentration per ml (and the total number of cells) can be determined using the following calculations:

CELLS PER ML = the average count per square x 10^6

TOTAL CELLS = cells per ml x the volume of cell suspension.

10 squares were used for counting of each sample and an average count taken.

2.2.3. ADDITION OF TEST COMPOUNDS TO ISOLATED HEPATOCYTES

2.2.3.1 Pre-Incubation for Glycogen Phosphorylase Assay.

The cells were suspended in a volume of buffer (Krebs-Henseleit medium containing 10 or 30mM glucose to simulate the control or diabetic condition) to give a final concentration of 3-5x10^6 cells/ml. 2ml aliquots of this suspension were then placed in a 20ml plastic scintillation vial and incubated at 37°C under 95% air and 5% CO_2 with reciprocal shaking at 90 cycles per min for 30min prior to the addition of drugs.

After pre-incubation as above, 500μl aliquots of cells were transferred to 1-ml microcentrifuge tubes containing 50μl of drugs at various concentrations (insulin at 10^{-10}-10^{-6}M dissolved in 0.1M HCl; test compounds at 10^{-10}-10^{-3}M dissolved in distilled water except for diazoxide which was dissolved in 0.1M NaOH) or vehicle (control) and incubated at 37°C in a dry Heating Block (Genesys Instruments). The incubations were terminated after 5min by dropping the tubes in liquid nitrogen (Figure 7. The tubes were then stored frozen at -70°C until they were assayed for glycogen phosphorylase activity as described below. Duplicate incubations were made for each concentration. The assay of glycogen phosphorylase activity was performed within 24h as there is some inactivation of the enzyme even at this low temperature.

2.2.3.2. Pre-Incubation for Androst-4-ene-3,17-dione Metabolism Assay

Isolated hepatocytes (10^7cell/ml) were plated out on sterile petri dishes (NUNCLON, Denmark, 9cm diameter) at a density of 3x10^5 cells/cm^2 in Krebs-Henseleit medium (final volume 7ml). The plates were swirled carefully to ensure even distribution of cells over the surface of the plates. 70μl of compound or hormone were added directly to the cell culture at concentrations 10^{-10} to 10^{-5}M for insulin and

THE METHOD OF

THE EFFECT OF DRUGS ON GLYCOGEN PHOSPHORYLASE a ACTIVITY IN ISOLATED RAT HEPATOCYTES

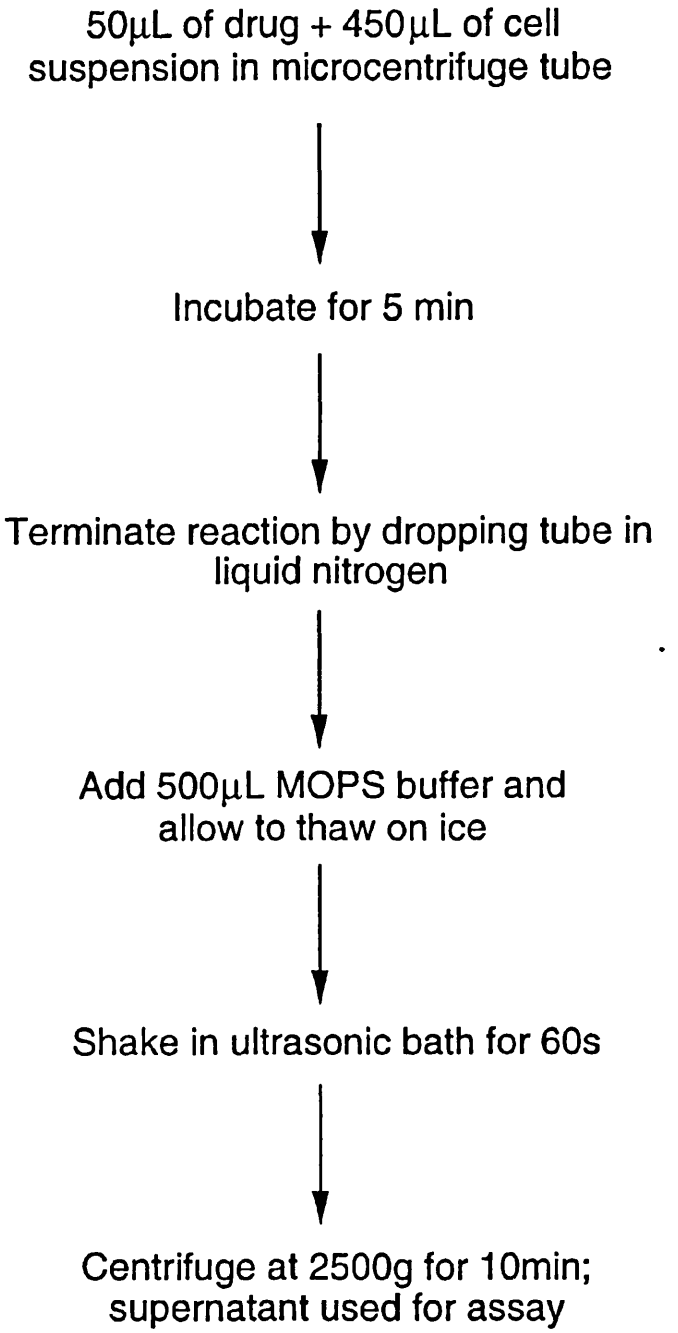


Figure 7. Illustration the method of the effect of drugs on glycogen phosphorylase a activity in isolated rat hepatocytes

10^{-10} to 10^{-5}M for the test compounds. Insulin was dissolved in 0.1M hydrochloric acid and the test compounds in distilled water (except for diazoxide, which was dissolved in 0.1M NaOH). The additions did not alter the pH of the medium. Controls were treated with a similar amount of vehicle. The plated cells were subsequently incubated at 37°C under 95% air and 5% CO_2 in a humidified incubator (Flow Laboratories) and shaken at 90 oscillation /min for 30min. At least six separate samples were taken for each control and test batch of cells.

2.2.4. ASSAYS

2.2.4.1 Glycogen Phosphorylase Assay

The assay of glycogen phosphorylase in this work depends on the reaction between D-glucose-1-phosphate (Glc-1-P) and glycogen in the reverse, unphysiological direction (i.e. in the direction of glycogen synthesis). The principle involves the incorporation of [^{14}C]glucose from Glc-1P into glycogen. The filter paper assay method of Tomas *et al* (1968), modified by Stalmans & Hers (1975) was used to determine the enzyme activity.

An equal volume (500µl) of ice-cold disruption buffer was added to the frozen samples from the pre-incubation step described above, allowed to thaw on ice and then shaken for about 60s in an ultrasonic bath. The mixture was centrifuged at 2500g at 5° C for 10 min in a Damon-IEC refrigerated centrifuge. 50µl of the supernatant was added to 50µl of assay mixture (for composition of assay mixture see below) in a microcentrifuge tube, mixed by means of a rotary mixer and incubated in a water bath at 37°C for 20min (Figure 8). The reaction was stopped by removing 30µl aliquots of the mixture and spotting it to filter paper pieces marked with the sample number in pencil (Whatman 3MM chromatography paper, 10mm^2), and dropping in a beaker containing 400ml of 66%(v/v) ethanol in water to precipitate the radioactively-labelled glycogen. Excess [U-^{14}C] glucose-1-phosphate will disperse into the ethanol/water.

Blank samples were prepared by mixing 50µl of disruption buffer with 50µl of assay mixture. The ethanol, mixed on a magnetic stirrer and screened from the paper squares with an aluminium mesh to prevent disintegration of the filter papers, was changed every 40min (3 times) to wash the filter papers. After the final wash the filter paper squares were mixed for 3min in acetone and dried in air. The radioactivity on each filter paper was measured in a Tri-Carb 2000CA scintillation counter (Packard, Berks; England), by placing the filter papers in 20-ml plastic scintillation vials with 2ml of Ecoscint scintillation (National Diagnostics, Atlanta, GA, U.S.A.). The total amount of radioactivity present in the assay mixture was measured by spotting 50µl of the assay bufer onto a filter paper, allowing to dry and counting as above. The method is illustrated in the flow diagram show on the following page.

2.2.4.2. Androst-4-ene-3,17-dione Metabolism Assay

The method first described by Gustafsson & Stenberg (1974) was used as modified by Hussin & Skett (1986). 7µl of [4-^{14}C]-androst-4-ene-3,17-dione ($1.5x10^5$ d.p.m, dissolved in acetone), 10µl of androst -4-ene -3,17-dione (100µg, dissolved in acetone) were added to each cell suspension and incubated for 30 minutes at 37°C in 95% air and 5% CO_2 in a humidified incubator whilst being shaken at 90 cycles/min . After the assay incubation, 50µl from each sample was transferred to a 1ml microcentrifuge tube to assay the amount of protein in each cell suspension. The rest of the sample (including the cells) was transferred to a test tube and the reaction was stopped by adding 5ml of Folch solution (chloroform: methanol , 2:1) to each sample, followed by 0.5ml of 0.9% NaCl solution to aid extraction of the substrate and its metabolites into the organic layer. Another 50µl (500µg) of androst-4-ene-3,17-dione was added , the tubes shaken gently and left to stand overnight in the dark for phase separation into organic and aqueous layers. In order to acheive complete separation

GLYCOGEN PHOSPHORYLASE ASSAY

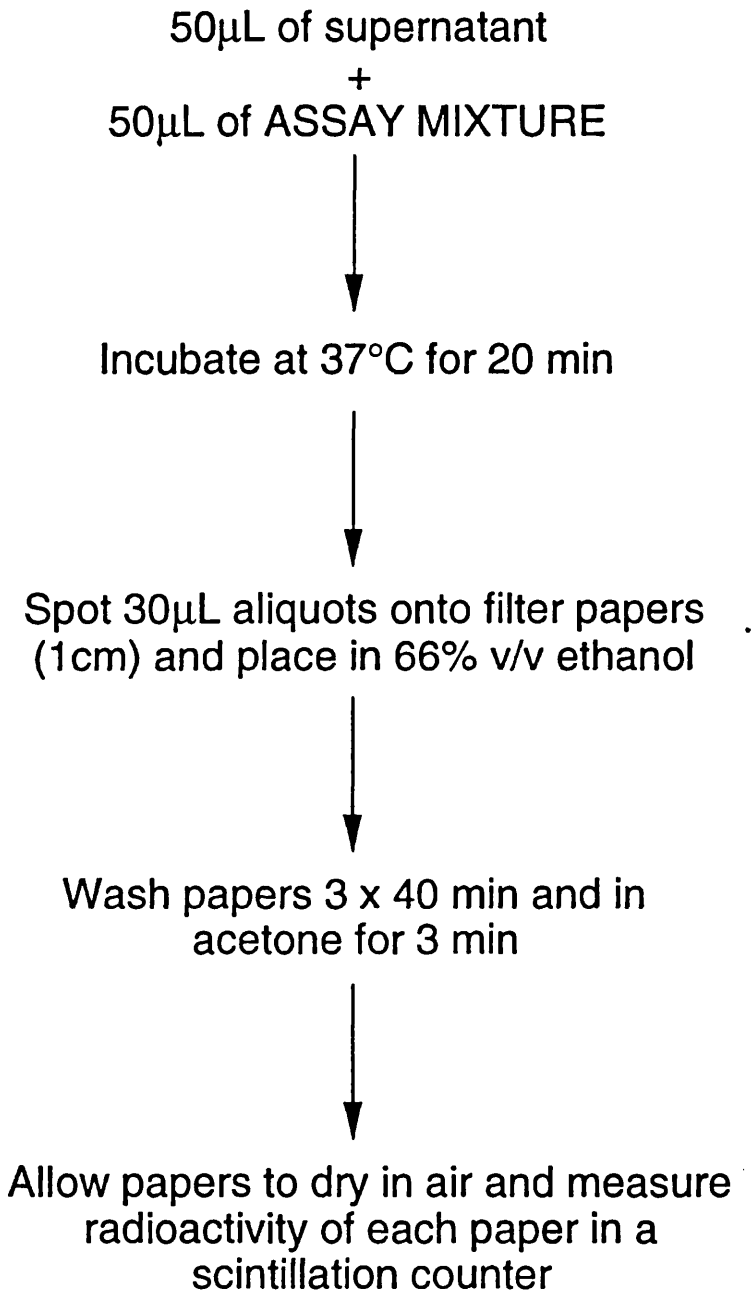


Figure 8. Illustration of glycogen phosphorylase assay

of the 2 phases, the tubes were centrifuged at 1500 g at 5°C for 5 min in a Damon-IEC refrigerated centrifuge. The lower organic layer was transferred to another tube and dried at 50°C under oxygen-free nitrogen (to prevent degradation of the metabolites by atmospheric oxygen) using a Techne Dri Block. The extract was redissolved in 5 drops of chloroform (in the ultrasonic water bath for at least 30 seconds). Individual samples were then spotted onto 250μm silica gel thin layer chromatography plates (20cm x 10cm , F-254, Merck, Darmstadt, Germany) and separation of metabolites was achieved by running the plates in a mobile phase consisting of chloroform : ethylacetate (4 : 1, v/v) in tanks equilibrated with the solvent for at least one hour before commencing the separation. The samples were spotted (4 samples on a plate) at the lower edge of the T. L .C. plate ($\approx$1.5 cm in), using a 10μl disposable micropipette. After the solvent front had run to about 1 cm from the top edge of the plates the plates were dried in warm air. Autoradiograph of the plates for 7 days with X-Omat film (Kodak, France) located each of the radioactive bands shown in Fig 9. The radiolabelled bands were scraped into polythene scintillation vials and suspended in Ecoscint liquid scintillant (National Diagnostics, Atlanta, GA, U.S.A.) and the amount of ^{14}C label present in each band was evaluated in a Packard Tri-carb 2000CA scintillation counter. The amount of each metabolite expressed as pmol metabolite formed per min per mg of protein was determined using the formula shown in the section on calculations and statistics (section 2.2.7.2)

It is apparent from Figure 9 that there are six main metabolites of androst-4-ene-3,17-dione which can be separated by this method. However, it has been shown that 3α/β-oxosteroid oxidoreductase does not metabolise the parent compound, but that metabolism by this enzyme occurs subsequent to metabolism by 5α-reductase. Hence, for the purpose of this study, only the activities of the enzymes metabolising the parent molecule were evaluated and hence the amount of metabolite produced by subsequent metabolism of 5α-androstane-3,17-dione (as produced by 5α reduction of androstenedione) was not determined separately but the amount of metabolite produced by this enzyme was added to the amount of unchanged 5α-androstane-3,17-

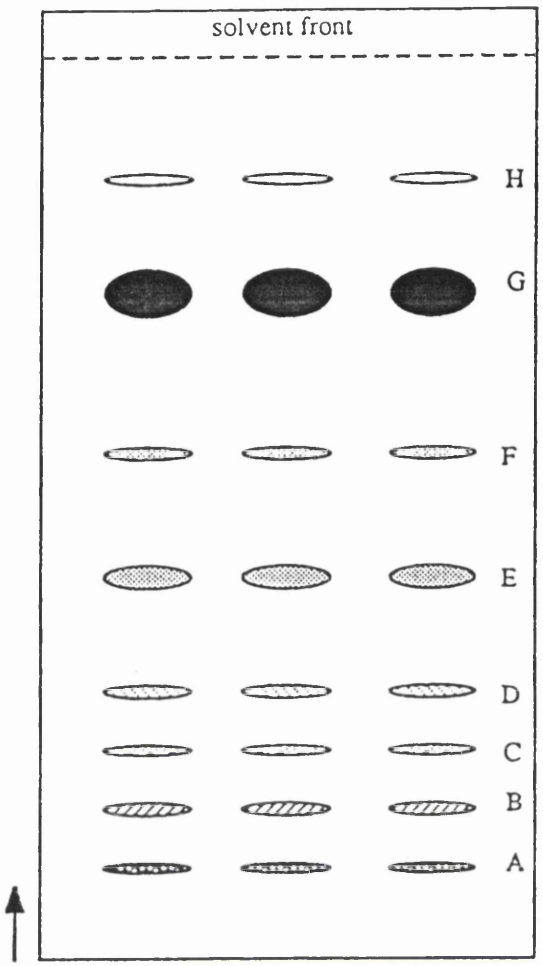


Figure 9. Schematic representation of androst -4-ene-3,17-dione metabolites separation by one-dimensional thin layer chromatography

specific bands identified by autoradiography are indicated :

A. Spotting band
B. androst-4-ene-7α-ol-3,17-dione
C. androst-4-ene-6β-ol-3,17-dione
D. androst-4-ene-16α-ol-3,17-dione
E. Testosterone / Epitestosterone
F. 5α-Androstane-3α(β)-ol-17-one
G. Unchanged substrate Androst-4-ene-3,17-dione
H. 5α-Androstane-3,17-dione

dione to determine the total amount of metabolite produced by the activity of 5α reductase.

2.2.5. ASSAY OF SERUM GLUCOSE

In the experiments where blood was required for detection of serum glucose level, the blood was collected from the tip of the tail after it had been cut under halothane / nitrous oxide anaesthesia. Serum was prepared by centrifugation at 3000 g for 3 minutes in an MES Microcentaur centrifuge. The upper serum layer was removed using a micropiptte and assayed for glucose by a techinque based on the glucose oxidase method (Carroll et al, 1970) using a kit supplied by Boehringer Mannheim GmbH.

2.2.6 PROTEIN ASSAY

The amount of protein present in the supernatant of the freeze-thawed cells or the assay mixture of the steroid assay was measured using the Folin-phenol method (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

2.2.7. CALCULATIONS AND STATISTICS

2.2.7.1 Glycogen Phosphorylase

The activity of glycogen phosphorylase in a given sample was calculated as the amount of ^{14}C-glucose incorporated in glycogen after subtraction of the blank values. Enzyme activity was calculated by the equation below and expressed as nmol of ^{14}C-glucose 1-phosphate incorporated into glycogen min^{-1} mg of $protein^{-1}$.

ENZYME ACTIVITY=$(DPM_S-DPM_B)/DPM_T$x[S]X(1/P)x(1/t)x(10,000/3)

where,

DPM_S, DPM_B, DPM_T, are the disintegrations/min for the sample, blank and total amount of radioactivity in the assay respectively.

[s]=substrate(GLU-1-*P*) concentration (50mM in all the assays)

p = protein conc.of the supernatant

t=incubation time(min)

2.2.7.2. Steroid Metabolism

The amount of each metabolite of androst-4-ene-3,7-dione expressed as pmole metabolite formed per minute per 10^6 cell or mg of protein was determined using the formula shown below:

$$\text{pmol metabolite /min/}10^6\text{ cell or mg of protein} = S \times \frac{Cm}{Ct} \times \frac{1}{t} \times \frac{1}{n} \times \frac{1}{MW}$$

S = amount of substrate added (μg)

Cm = d.p.m. metabolite

Ct = d.p.m.total

t = incubation time (minutes)

n = number of cells (divided by 10^6) or mg of protien

MW = molecular weight of substrate (280)

Results were expressed a % of the relevant control. Mean and standard deviations were calculated using a custom-made computer program and statistical analysis was performed using ANOVA followed by Dunnet's test. A p value less than 0.05 was considered statistically significant.

2.2.8. SOURCES OF HORMONES, DRUGS AND REAGENTS

Hormones, chemicals and reagents are listed according to the manufacturer.

Sigma Chemical Co.Ltd., St.Louis, USA

MOPS

DL-dithiothreitol

caffeine

rabbit liver glycogen Type III

HEPES

Saccharin

Phenformin

Diazoxide

Melamine

Metformin.

Insulin (porcine)

EGTA

Streptozotocin

Bovine serum albumin

4-Androstene-3,17-dione

α-D-Glucose-1-phosphate

Hopkins &Williams,.Essex, England:-

sodium fluoride

disodium EDTA

British Drug Houses, Poole,England

Folin-Ciocalteu's phenol reagent

Amersham International p.l.c., Bucks, England:

D-[U-^{14}C]Glucose-1-phosphate, potassium salt

[4-^{14}C]androst-4-ene-3,17-dione

Boehringer,Mannheim, Germany:

Collagenase A form Clostridium histolyticum

Glucose assay kit

Fisons,Loughborough, England

salts, for physiological solutions and buffers.

NaCl, KCl, $MgSO_4$, KH2PO_4, $NaHCO_3$, Glucose.

Aldrich Chemical Co-Ltd

3,5-Diamino-1,2,4-triazole

1-phenyl biguanide hydrochloride

1,3,5-Triazine

Kodak, France

X Ray film (X-Omat)

Developer (DX 24)

Fixer (FX-40)

2.2.9. BUFFERS AND PHYSIOLOGICAL SOLUTIONS

2.10.1 Krebs-Henseleit buffers

a)Ca^{2+}Free Krebs-Henseleit solution

	mM
NaCl	117.0
KCl	4.7
$MgSO_4$	1.2
KH_2PO_4	1.2
$NaHCO_3$	2.4
HEPES	10.0
Glucose	11.1

The above were dissolved in distilled water and the pH adjusted to 7.4 with 0.1M NaOH.

b) Collagenase buffer

As above (a) but with $CaCl_2$(4.2mM) and the pH adjusted to 7.6.

c) Washing buffer

As in(a) but with $CaCl_2$(1.3mM)

d) Cell disruption buffer

	mM	g/100ml
MOPS	100	2.090
NaF	200	0.840
EDTA	30	1.120
DTT	10	0.154

store at 4°C

e)Glycogen phosphorylase assay mixture

		mg/10ml
Caffeine	1mM	1.942

Glycogen	2%	200
D-Glucose-1 phosphate	100mM	336.3
D-[U^{14}C]Glucose-1 phosphate	50nmol	25µl
(sp.activity: 284mCi/mmol)		
Stored at -20°C		

RESULTS

3.1 SYNTHESIS OF BIGUANIDES AND GUANAMINES.

The authenticity of the synthesised compounds was confirmed by melting point measurement using the Kofler hot stage apparatus. The melting points agreed with the literature values (Overberger et al; 1954; Shapiro et al; 1957). Microanalysis for C, H and N were performed using a Carlo Erba Analyser and the products gave values in agreement with theory. ^{1}H, ^{13}C-NMR spectra were recorded with Bruker AM 200SY and WP 200SY spectrometers. Spectra were consistent with the proposed compounds.

3.1.1. NMR Spectra of Substituted Guanamines and Biguanides

Since the guanamines might be protonated when bound to receptors, the NMR spectra of the hydrochlorides were examined in the polar solvent $(CD_3)_2SO$ to gain insight into the sites of protonation and the shapes of the cations.

The ^{1}H and ^{13}C spectra of 2-amino-4-phenethylamino-1,3,5-triazine hydrochloride showed that protonation had occurred on the triazine ring rather than on either of the amino substituents. The amino and alkylamino groups were planar rather than pyramidal and rotation about the C(2)-N and C(4)-N bonds was slow on the NMR time scale. The NH_2 protons gave separate signals and the phenethyl side chain gave rise to a pair of geometrical isomers (rotamers) in a ratio *ca.* 3:1. Thus the 4-NH proton gave triplets (*J* 5.7 Hz) at δ 9.16 (major rotamer) and 9.06 (minor rotamer). Other pairs of strong and weak signals were observed, respectively, at δ 8.75 and 8.51 (br s, N*H*H), 8.40 and 8.48 (s, 6-H), 8.37 and 8.10 (br s, NH*H*) and 3.54 and 3.58 (q, CH_2NH). The phenyl group gave a multiplet at δ 7.27 and the phCH_2 group a triplet (J 7.4 Hz) at δ 2.87. No signal was discernible for the triazine ring NH, nor for water in the solvent. Apparently, intermolecular exchange of this proton between one or more of the triazine nitrogens and the water had caused extensive signal broadening. Pairs of

strong and weak signals in the ^{13}C spectrum confirmed the presence of major and minor rotamers.

The spectra of 2-amino-4-benzylamino-1,3,5-triazine hydrochloride also indicated the presence of rotamers (ratio *ca.* 3:1) arising from protonation of the triazine ring. Pairs of strong and weak signals were again observed, respectively, at δ 9.57 and 9.48 (t, *J* 6 Hz, CH_2NH), 8.78 and 8.60 (br s, N*H*H), 8.44 and 8.51 (s, 6-H), 8.40 and 8.11 (br s, NH*H*), and 4.55 and 4.60 (d, *J* 6.2 Hz, CH_2). The phenyl group gave a muliplet at δ 7.34. Again, no signals were observed for the triazine ring NH or for water in solvent. The ^{13}C spectrum confirmed the presence of major and minor rotamers.

The spectra of the hydrochloride of the more weakly basic 1-amino-4-phenylamino-1,3,5-triazine differed from those of the foregoing 4-alkylamino compounds. Signals for only one species were apparent, although the presence of two, distinct NH2 signals again showed that the triazine ring was protonated and that rotation of the planar NH2 group was slow on the ^1H NMR time scale. Also, a signal for the triazine ring NH was observed as a very broad singlet at δ *ca.6.* This signal integrated for almost 2H, indicating exchange with solvent water. Apparently, rotation about the C(4)-N bond occurs as a result of only weak electron release from the anilino nitrogen. The ^1H spectrum showed δ 11.0 (br s, PhN*H*), 8.90 (br s, N*H*H), 8.56 (s, 6-H), 8.44 (br s, NH*H*), 7.73 (d, *J* 7.7 Hz, *o*-Ph-H), 7.39 (t, *J*7.7Hz, *m*-Ph-H), 7.22 (t, *J*7.3 Hz, *p*-Ph-H) and *ca.* (br s, NH).

The spectra of the hydrochlorides of phenethyl- and benzyl- biguanide were simpler. Only one species was observed for each salt and signals for only two kinds of NH proton were apparent. Thus, the phenethylbiguanide (phenformin) hydrochloride gave δ 7.62 (t, J5.4Hz, CH_2NH), 7.27 (m,Ph), 7.05 (Br s, NH), 3.34 (q, *J*6.8 Hz, CH_2NH) and 2.77 (t, *J*7.4, PhCH_2) while the benzyl derivative gave δ 7.96 (t, *J*6.0, CH2N*H*), 7.32 (*m*, Ph), 7.07 (br s, NH) and 4.36 (d, *J*6.0, CH_2). The broad, NH singlets at δ 7.05 and 7.07 each integrated approximately for 6 protons. presumably, protonation occurs on one or other of the C=NH groups, but the signals for the 6 distinct protons on the unsubstituted nitrogens give only on broad singlet.

3.2. The effect of biguanides, guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in normal rat hepatocytes

Figures 10 and 11 show the effects of all of the compounds tested on the activity of glycogen phosphorylase a in isolated normal rat hepatocytes, in comparison to the control (100%). Insulin was used as standard in the experiments in order to investigate the insulin-like activity of the synthesised compounds as potential anti diabetic agents. Phenformin was used to test the efficacy of the synthesised compound against a known oral hypoglycaemic agent .

3. 2. 1. The effect of phenethylbiguanide and phenethylguanamine.

In Figure 10A phenethylbiguanide (phenformin) in the concentration range of 10^{-10}M to 10^{-3}M caused a significant decrease in the activity compared to control (basal phosphorylase activity 17.8 ± 1.3 nmol/ mg of protein/ min, $p< 0.01$) although the effect did not seem to be dose - related. The effect was more pronounced with phenethylguanamine (Figure 10A) at 10^{-6}M, 10^{-7}M,10^{-8}M and 10^{-10}M (which diminished the activity significantly to 35%-48% of control ($p<0.01$). Similarly, significant inactivation of glycogen phosphorylase a was observed at all insulin concentrations studied (10^{-10}M to 10^{-5}M) with approximately maximum activity at physiological concentration 10^{-9}M (15% of control; $p<0.01$). In this experiment on the same cells, the insulin was more effective than phenethylbiguanide or phenethyguanamine ($p< 0.001$) except at low concentration 10^{-10}M when there was no significant difference between insulin and phenethylguanamine.

3. 2. 2. The effect of phenylbiguanide and phenylguanamine.

Both phenylbiguanide and phenylguanamine at concentrations between 10^{-10}M and 10^{-4}M caused a dose-dependent decrease in enzyme activity (Figure 10B). It was seen that insulin had more effect on the activity (inhibition to 6% of control) when compared to phenylbiguanide and phenylguanamine ($p< 0.001$). There was no difference seen between the effects of phenylbiguanide and phenylguanamine.

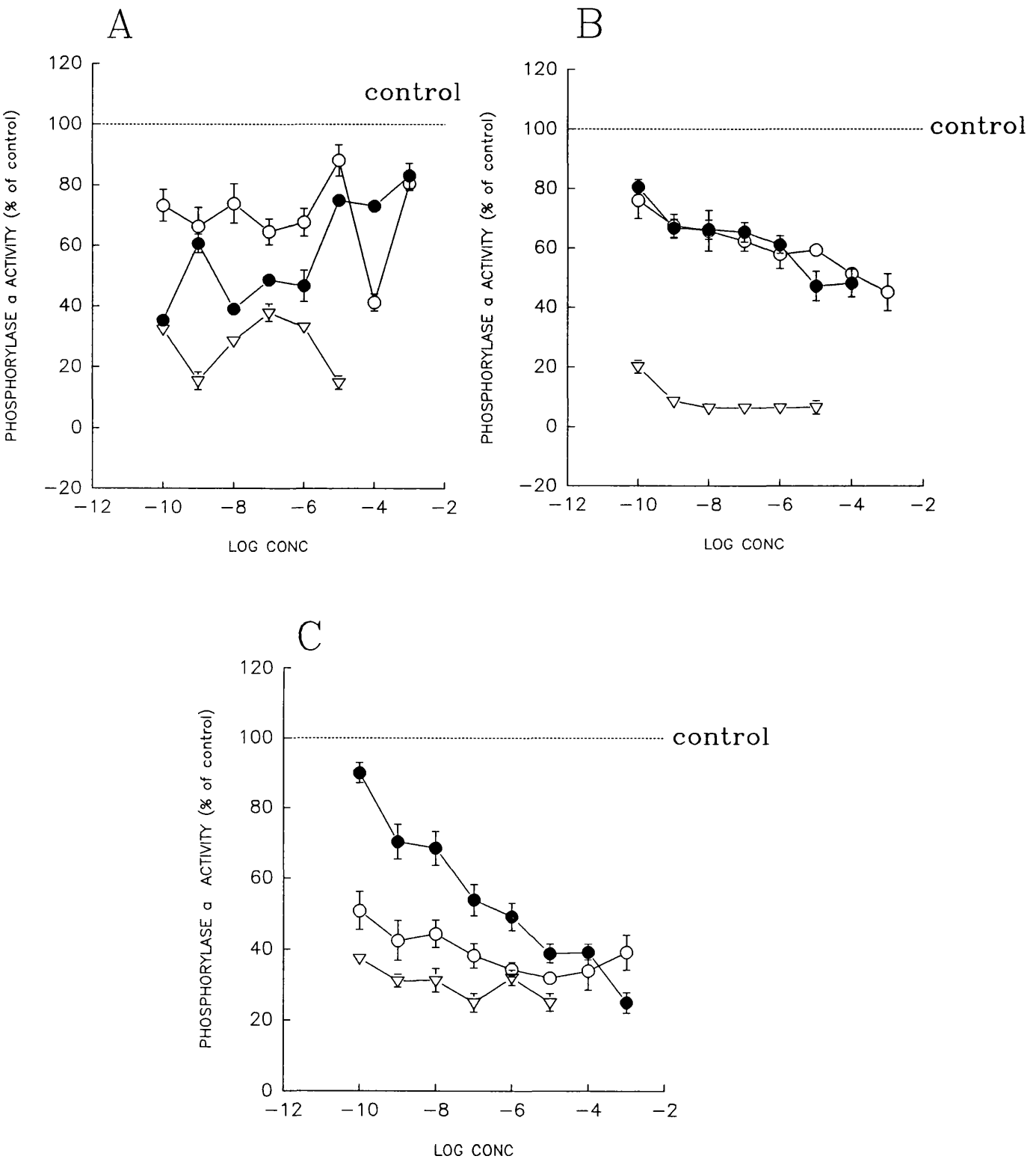
3. 2. 3. The effect of benzylbiguanide and benzylguanamine.

Benzylbiguanide, exhibited a significant inactivation of glycogen phosphorylase a (to 30% of control) a similar decrease to that seen with insulin (Figure 10C). The cyclization of benzylbiguanide to benzylguanamine did not improve the activity on glycogen phosphorylase, indeed there was less of an effect at lower concentrations.

3. 2. 4. The effect of phenethylbiguanide and 1,1- dimethylbiguanide

To show in which part of the chemical structure the activity resides, a comparison was made of phenethylbiguanide (phenformin) and 1,1-dimethylbiguanide (metformin) which belong to the same chemical class of anti diabetic drugs. As shown in Figure 11A, both compounds significantly inactivate phosphorylase a in comparison to control (100%) ($p < 0.01$; basal phosphorylase activity 47.7 $\pm$ 2.3 nmol/ mg of protein/ min). The cells were more sensitive to metformin than phenformin with a greater effect occurring at most concentrations below 10^{-4}M and the greatest effect occurring at 10^{-6}M ($p< 0.001$, compared to phenformin). Neither compond had an effect as marked as insulin ($p<0.001$).

Figure 10: Effect of various concentrations of (A) phenethylbiguanide (O) and phenethylguanamine (●), (B) phenylbiguanide (O) and phenylguanamine (●), (C) benzylbiguanide (O), benzylguanamine (●) and insulin (∇) on glycogen phosphorylase a activity in normal rat hepatocytes compared to control (100%). The cells were pre incubated for 30 min in Krebs-Henseleit medium and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All value wre signficantly different from control, $p < 0.01$. Basal phosphorylase activity for control in graph A (17.8 ± 1.3 nmol/ mg of protein/ min), B (29.1 ± 1.8 nmol/ mg of protein/ min) and C (28 ± 1.5 nmol/ mg of protein/ min)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PHOSPHORYLASE a ACTIVITY (% of control)
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3. 2. 5. The effect of phenethylbiguanide and saccharin.

Further examination was made of two chemical classes of potential hypoglycaemic agents containing N-groups and S-groups, phenethylbiguanide (phenformin) containing an amino group and o-sulfobenzimide (saccharin) containing a sulphur group. From the concentration response curve for glycogen phosphorylase a inactivation (Figure 11B), it is seen that both drugs significantly inactivated the enzyme at concentration of 10^{-10}M-10^{-3}M (52% - 81%) compared to control ($p<0.01$; basal phosphrylase activity 38.2 $\pm$ 1.7 nmol/ mg of protein/ min). It was observed that phenformin (phenethylbiguanide) was equally as effective in inactivating the glycogen phosphorylase a as saccharin (o-sulfobenzimide) at most concentrations. Neither compound was as effective as insulin in inactivating glycogen phosphorylase a.

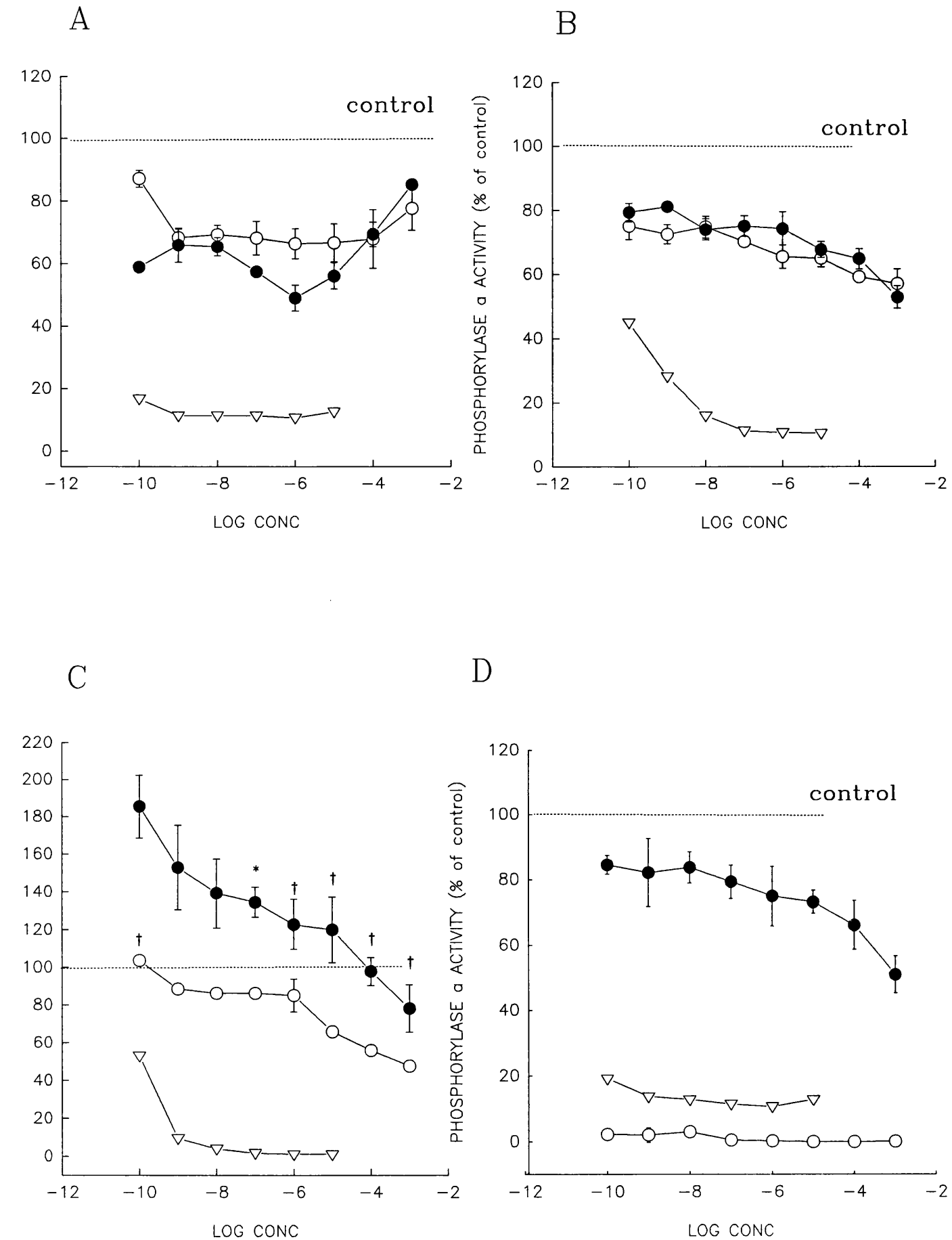
3. 2. 6. The effect of saccharin and diazoxide

The effect of saccharin, whose structure contains an amino group plus a sulphur group in a 5-membered ring, was compared with diazoxide (a hyperglycaemic drug), which contains the same groups in a 6-membered ring . Saccharin significantly decreased the activity of glycogen phosphorylase a at 10^{-9}M - 10^{-3}M (47% - 88%, of control; $p< 0.01$; basal phosphorylase activity 29.5 $\pm$ 1 nmol/ mg of protein/ min) whereas diazoxide caused the enzyme to respond in the opposite manner with a significant increase in the activity of glycogen phosphorylase at 10^{-10}M to 10^{-8}M ($p< 0.01$) (Figure 11C). Again insulin was more effective than either saccharin or diazoxide.

3. 2. 7. The effect of melamine and 3,5 -diaminotriazol

In addition melamine, an example of a triazine with a 6-membered ring with 3 amino groups, and 3,5-diaminotriazole, which contains a 5-membered ring were used .

Figure 11. The effect of (A) phenethylbiguanide (phenformin) (O) and 1,1-dimethylbiguanide (metformin) (●), (B) phenethylbiguanide (O) and sulfobenzimid (saccharin) (●), (C) sulfobenzimide (saccharin) (O), 7 chloro-3 methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide) (●), (D) 2,4,6-triamino-1,3,5-triazine (Melamine) (O), 3,5-diamino triazol (●) and insulin (∇) on phosphorylase a activity in normal rat hepatocytes in comparison to control (100%). Hepatocytes were pre incubated for 30 min in Krebs-Henseleit medium and the effect of 5 min expsure to the compounds and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test $p < 0.01$ compared to basal phosphorylase activity (100%). All values are significant at $p<0.01$, exapet, * $p < 0.05$ and † (not significant). Basal activity of glycogen phosphorylase for control in graph A (47 ± 2.3 nmol/ mg of protein/ min), B (38 ± 1.7 nmol/ mg of protein/ min), C (29 ± 0.96 nmol/ mg of protein/ min), D (47 ± 2.2 nmol/ mg of protein/ min).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†

Melamine completely inhibited enzyme activity but this was shown to be due to a decreased viability of the cells (Figure 11D). However 3,5-diaminotriazole significantly inactivated the enzyme in a dose- dependent manner with the maximum effect at 10^{-3}M (51% of control; $p< 0.01$; basal phosphorylase activity 47.7 $\pm$ 2.2 nmol/ mg of protein/ min). Insulin was more effective than 3,5-diaminotriazole.

3. 3. The effect of streptozotocin - induced diabetes and insulin supplementation on glycogen phosphorylase a in rat liver.

Experiments were carried out with hepatocytes from control, diabetic and insulin treated diabetic animals. In *in vivo* studies, streptozotocin-induced diabetes resulted in a 52% decrease in glycogen phosphorylase a activity. When insulin was replaced in these diabetic rats glycogen phosphorylase a activity returned to its control values. The basal activity of phosphorylase a in the liver of the three groups of animals ($n = 8$) is shown Figure 12. It was significantly lower in diabetic animals (basal phosphorylase activity 19 $\pm$ 2.6 nmol/mg of protein/min, $p< 0.01$) than in the control (basal phosphorylase activity 36 $\pm$ 8.3 nmol/ mg of protein/ min) or insulin treated diabetic animals (basal phosphorylase activity 31 $\pm$ 1.1nmol/ mg of protein/ min). No significant difference between the control and the insulin-treated diabetic rats was found.

3. 4. Effect of biguanides, guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in cultured hepatocytes from STZ diabetic rats.

The possibility that phenethylbiguanide and phenethylguanamine act like insulin on glycogen phosphorylase a activity in isolated hepatocytes from diabetic rats was explored. The basal activity of glycogen phosphorylase a in these hepatocytes was 8.8

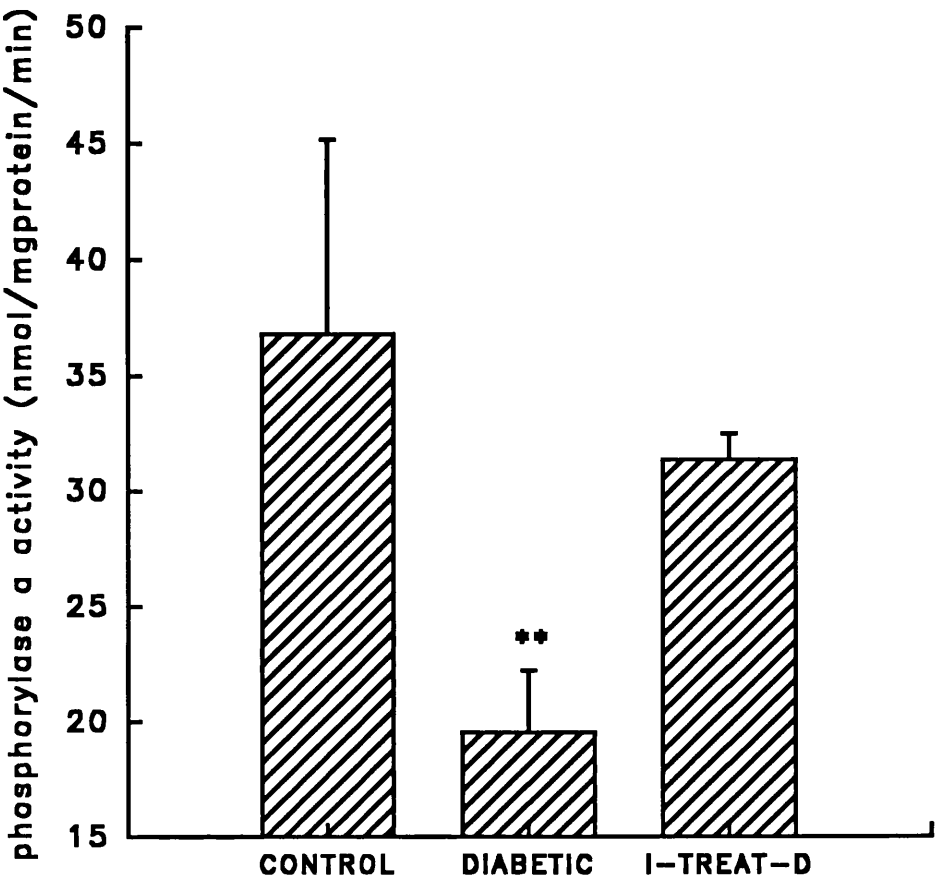


Figure 12. Glycogen phosphorylase a activity in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers. Values are the mean ± s.d. for eight animals. P values relative to the control values were obtained by ANOVA followed by Dunnet's test,**$p < 0.01$. Basal phosphorylase activity for control: 36 ± 8.3 nmol/ mg of protien/ min, for diabetic 19 ± 2.6 nmol/ mg of protein/ min and for insulin treated diabetic 31 ± 1.1 nmol/ mg of protein/ min.

± 0.4 nmol/ mg of protein/ min. This is very much lower than the basal activity found in control cells and is consistent with the data presented above in the *in vivo* experiments.

3. 4. 1. The effect of phenethylbiguanide and phenethylguanamine.

Exposure of diabetic rat hepatocytes to phenethylbiguanide for 5min resulted in a significant increase in glycogen phosphorylase a (Figure 13A). This increase occurred at all concentration of phenethylbiguanide with the maximum effect at 10^{-9}M (282% compared with the control). The cyclization of phenethylbiguanide to phenethylguanamine did not change the overall activation of phosphorylase a except that there was a tendency for diabetic hepatocytes to be more responsive to phenethylguanamine than phenethybiguanide at low concentrations (10^{-8} to 10^{-10}M). This effect was statistically significant ($p<0.001$, $p<0.01$, $p<0.05$ at the three concentrations respectively). The effect of insulin was not as pronounced as that seen with phenethylbiguanide or phenethyguanamine. Insulin stimulated glycogen phosphorylase in a dose dependent manner with maximal effect at 10^{-6}M (275%) as shown in Figure 13A.

3. 4. 2. The effect of phenylbiguanide and phenylguanamine

To further investigate whether the change in R- group had any effect on the activity of the glycogen phosphorylase, the effect of phenylbiguanide and phenylguanamine on the cells from diabetic rats was examined and compared to the effect of insulin. Figure 13B shows there is only a small change in glycogen phosphorylase a activity with phenylbiguanide at all concentrations (up to 175% of control) and the cyclization of phenylbiguanide to phenylguanamine gave only a small

change in activity. The hepatocytes were more responsive to insulin than either of the phenyl derivatives at higher concentrations.

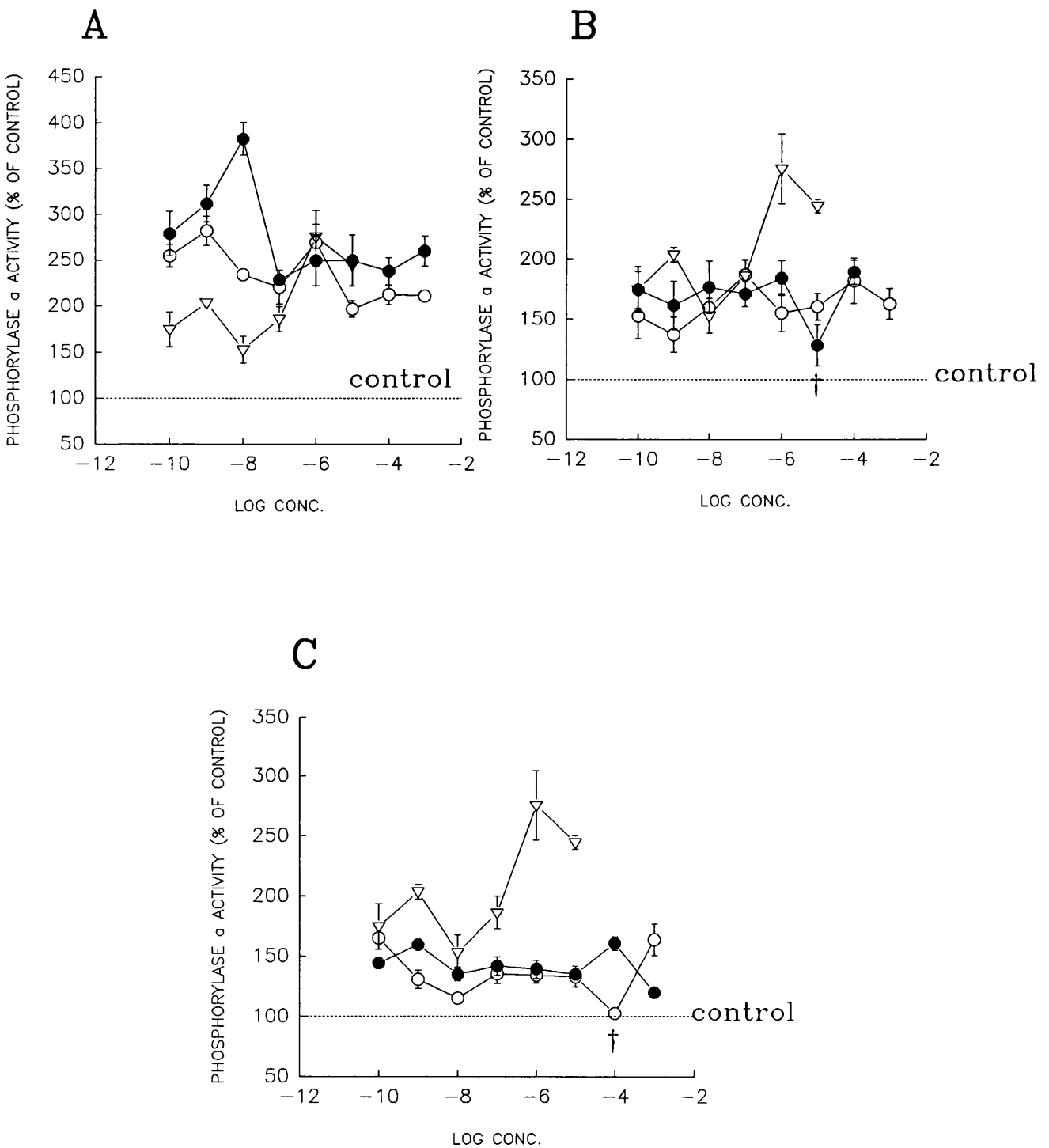
3. 4. 3. The effect of benzylbiguanide and benzylguanamine.

Examination of the effect of another R- group on the activity of glycogen phosphorylase in diabetic hepatocytes, showed that the replacement of the phenethyl group by the benzyl group (Figure 13C) again resulted in a less effective compound. Benzylbiguanide had minor effects although the effect was significant on glycogen phosphorylase a activity (to 160% of control, $p< 0.01$). When the benzylbiguanide was cyclized to benzylguanamine there was little change in activity of the compound. The phenethyl derivatives are therefore the most effective of all the compounds tested on diabetic hepatocytes.

3. 4. 4. The effect of phenethylbiguanide and 1,1- dimethylbiguanide

Both phenethylbiguanide (phenformin) and 1,1-dimethylbiguanide (metformin) were added to the diabetic hepatocytes in order to determine which had more effect on the diabetic state, Phenethylbiguanide (phenformin) increased the activity significantly compared to the control (100%) ($p<0.01$; basal phosphorylase activity 13 $\pm$ 1.6 nmol of ^{14}C-glucose 1-phosphate/ mg of protein/ min) at all concentrations except at 10^{-8}M where the activity was significantly increased at a significance level of ($p<0.05$) (Figure 14A). The maximum effect of phenethylbiguanide was observed at a concentration of 10^{-7}M-10^{-6}M. Attenuation of phenethylbiguanide's effect could be seen at concentrations of 10^{-5}M and 10^{-4}M. Exposure of diabetic hepatocytes to 1,1-dimethylbiguanide (metformin) at different concentrations significantly increased enzyme activity except at 10^{-7}M. In diabetic rat hepatocytes, insulin gave a dose-dependent increase in enzyme activity. In contrast to the cells from normal rat hepatocytes, the cells from the diabetic animal were more responsive to

Figure 13. Increase in glycogen phosphorylase a activity in hepatocytes from a diabetic rat 5 min after stimulation by (A) phenethylbiguanide (○) and phenethylguanamine (●), (B) phenylbiguanide (○) and phenylguanamine (●), (C) benzylbiguanide (○), benzylguanamine (●) and insulin (▽) in comparison to control (100%). The cells were pre incubated for 30 min in Kerbs-Henseleit medium before exposure to the compounds and hormone. Values are the mean ± s.d of six experiments, analysed by ANOVA followed by Dunnett's test $p < 0.01$ compared to basal phosphorylase activity (100%). All values were significant at $p < 0.01$ except †= not significant. Basal phosphorylase activity in graph A, B, C: 8.8 ± 0.4 nmol/ mg of protein/ min.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phenethylbiguanide at various concentrations than to 1,1-dimethylbiguanide (metformin) ($p< 0.001$).

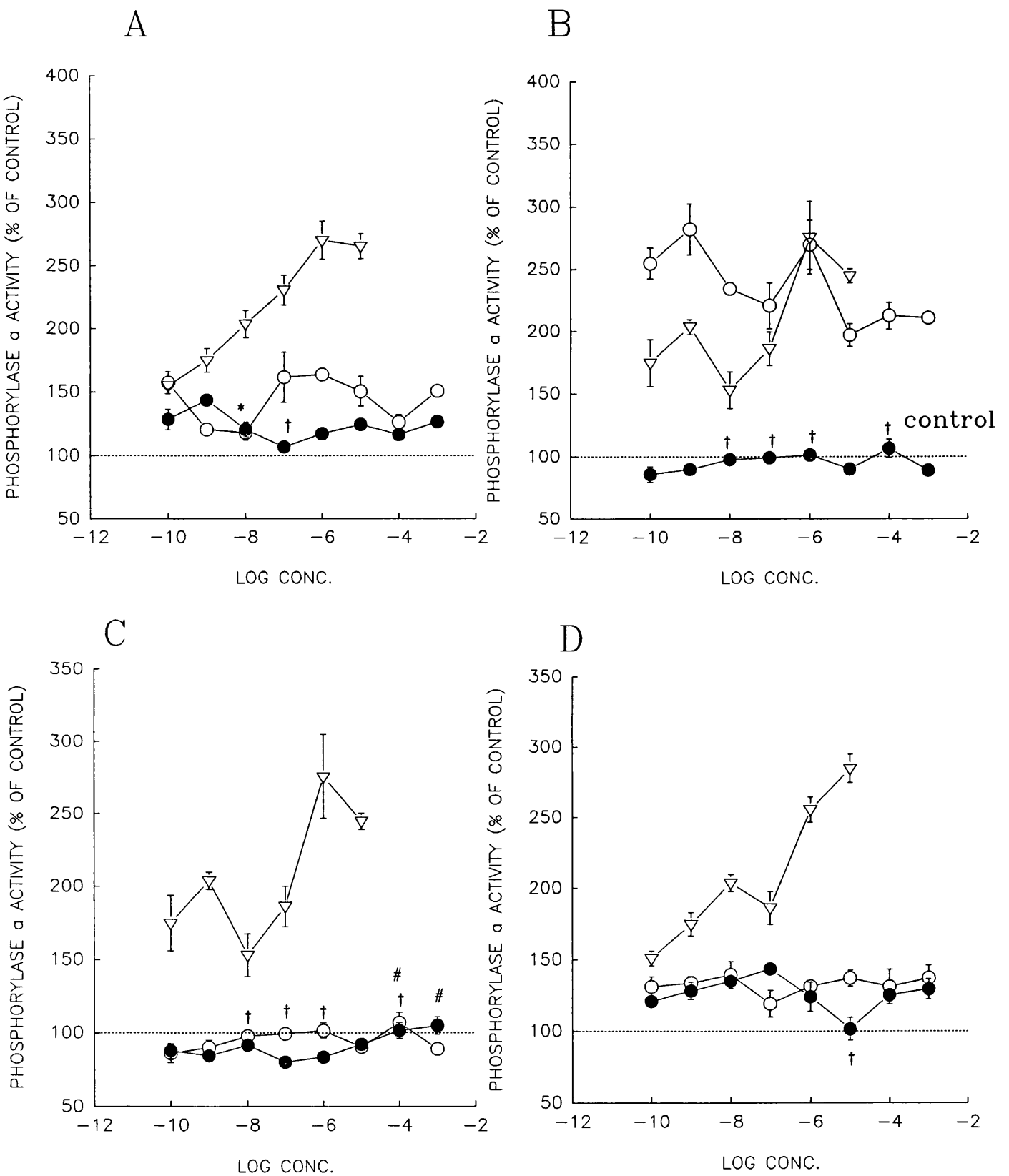
3. 4. 5. The effect phenethylbiguanide and saccharin.

In contrast to hepatocytes from normal rats which exhibit a dose-dependent decrease in glycogen phosphorylase activity with all concentrations of phenethylbiguanide (phenformin) and o-sulfobenzimide (saccharin), with diabetic hepatocytes there is a significant difference in the activity of phenethylbiguanide and saccharin. Saccharin at low concentrations (10^{-10} M and 10^{-9}M) and at high concentration (10^{-3}M, 10^{-5}M) decreased activity to 85% of control ($p< 0.01$, basal phosphorylase activity 8.8 $\pm$ 0.4 nmol/ mg of protein/ min), whereas phenethybiguanides gave the expected increase (Figure 14B).

3. 4. 6. The effect of saccharin and diazoxide.

In a comparison between saccharin (which contains an amino group plus a sulphur group in a 5 member ring) and diazoxide (which contains the same groups but in a 6 member ring) , it is seen that both compounds, when added to the same diabetic hepatocytes, gave few significant changes in enzyme activity (Figure 14C). When diazoxide was added at high concentration (10^{-4}M and 10^{-3}M), there was no significant change in the activity. A significant decrease in enzyme activity was, however, seen at 10^{-5}M diazoxide ($p<0.05$). At 10^{-10}M to 10^{-6}M diazoxide, the activity of the enzyme was reduced to significantly below the control ($p<0.01$). The maximum effect in the reduction of the activity was observed at a concentration 10^{-7}M diazoxide (80% of control). Saccharin only gave significant reduction in enzyme activity at 10^{-10}, 10^{-9}, 10^{-5} and 10^{-3}M.

Figure 14. Effect of different concentrations of (A) phenethylbiguanide (phenformin) (O) and 1,1-dimethylbiguanide (metformin) (●), (B) phenethybiguanide (phenformin) (O) and sulfobenzimide (saccharin) (●), (FC) sulfobenzimide (saccharin) (O) and 7-chloro-3 methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide) (●) (D) s-triazine (O) and 3,5-diamino triazol (●) and insulin (∇) on glycogen phosphorylase a activity in isolated rat hepatocytes from diabetic rats in comparison to control (100%). Hepatocytes were pre incubated for 30 min in Krebs-Henseleit medium and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Values are the mean ± s.d of six experiments, analysed by ANOVA followed by Dunnett's test, compared to basal phosphorylase activity (100%), all values were significant at $p<0.01$ except $*p< 0.05$, † and # = not significant.` Basal phosphorylase activity of glycogen phosphorylase for control in graph A (13 ± 1.6 nmol/ mg of protein/ min), B (8.8 ± 0.4 nmol/ mg of protein/ min), C (8.8 ± 0.4 nmol/ mg of protein/ min), D (10 ± 2.3 nmol/ mg of protein/ min).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3. 4. 7. The effect of s-triazine and 3,5-diaminotriazol.

In a further investigation to ascertain which part of the molecule is needed for activity, s-triazine, (containing a 6-membered ring) was compared with 3,5-diaminotriazol (a 5-membered ring). The results indicated that the activity of glycogen phosphorylase a was significantly stimulated by both 3,5-diaminotriazol and s-triazine in the concentration range 10^{-10}M to 10^{-3}M (120-137% of control, $p<0.01$; basal phosphorylase activity 10 $\pm$ 2.3 nmol/ mg of protein/ min) (Figure 14D). The responsiveness to the different concentrations of compounds was not as marked as with insulin.

3. 5. Effect of biguanides, guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in isolated hepatocytes from insulin-treated diabetic rats

The Figures 15 and 16 show the effects of the compounds tested on the activity of glycogen phosphorylase a in hepatocytes isolated from insulin-treated diabetic rats in comparison to control (100%).

3. 5. 1. The effect of phenethylbiguanide and phenethylguanamine.

In Figure 15A, phenethylbiguanide (phenformin) in the concentration range of 10^{-3}M to 10^{-8}M, showed a significant decrease in the activity to 59-88% of control ($p<0.01$ basal phosphorylase activity 32 $\pm$ 1.4nmol/ mg of protein/ min). When phenethylbiguanide was cyclized to phenethylguanamine the activity of phosphorylase a only diminished at 10^{-4}-10^{-7}M, ($p<0.01$) and at a low concentration of 10^{-9}M ($p<0.05$). The insulin was more effective than phenethylbiguanide or phenethylguanamine ($p< 0.01$).

3. 5. 2. The effect of phenylbiguanide and phenylguanamine.

Similarly, significant phosphorylase inactivation was observed at all phenylbiguanide and phenylguanamine concentrations studied between 10^{-10}M to 10^{-3}M compared to control ($p< 0.01$; basal phosphorylase activity 37 $\pm$ 6.5 nmol/ mg of protein/ min) although little evidence of dose- dependence was seen (Figure 15B). A comparison with insulin showed that the hormone had more effect on the activity (inhibition to 20% of control) when compared to phenylbiguanide and phenylguanamine ($p< 0.001$).

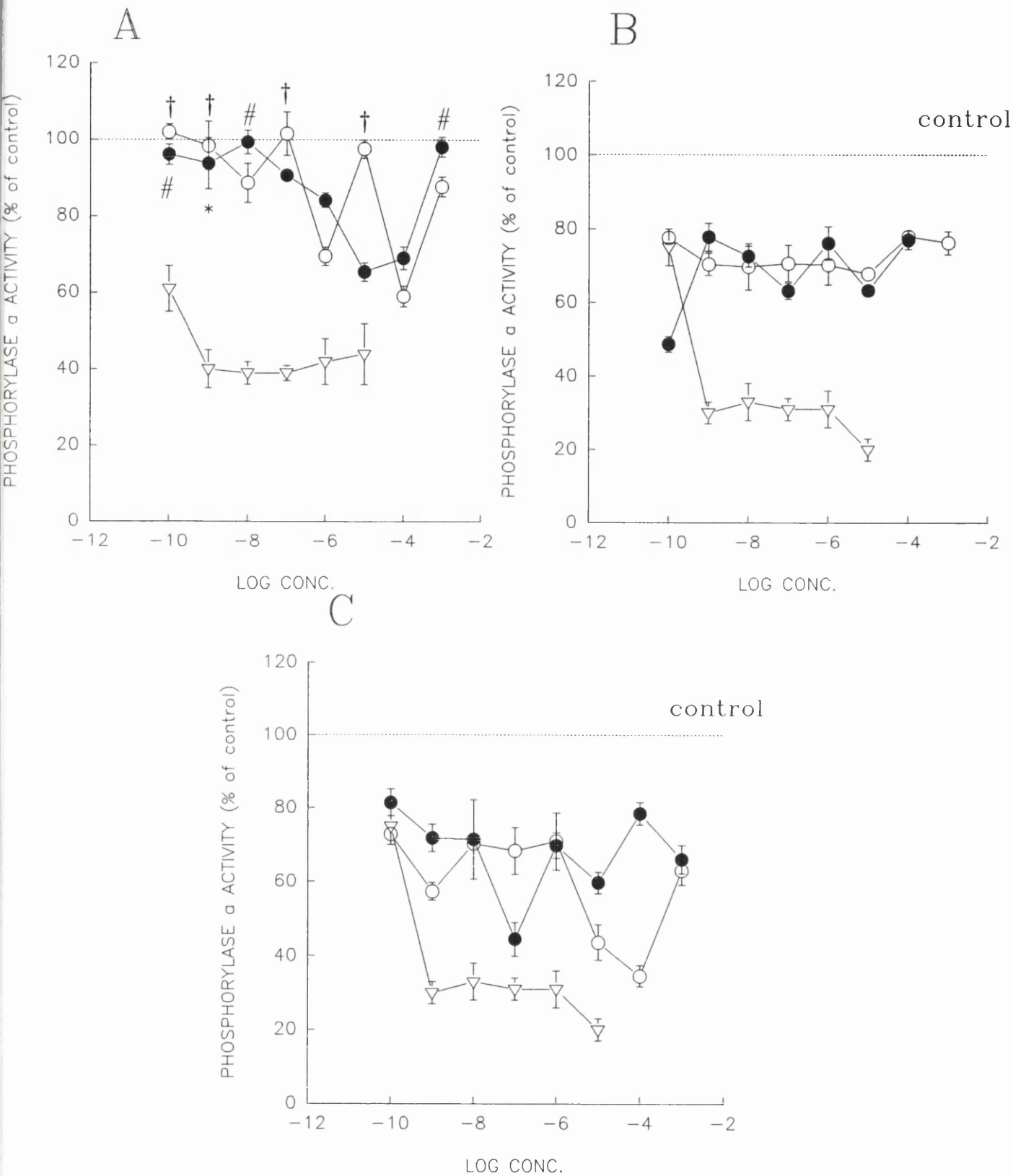
3. 5. 3. The effect of benzylbiguanide and benzylguanamine.

In Figure 15C, benzylbiguanide at concentrations between 10^{-10}M and 10^{-3}M decreased enzyme activity ($p< 0.01$; basal phosphorylase activity 34 $\pm$ 3.5 nmol/ mg of protein/ min). Maximum activity was observed at the high concentration of 10^{-4}M . Insulin had more effect on the activity except at low concentrations where there is no significant difference between insulin and benzylbiguanide. There was no overall improvement in the activity when benzylbiguanide was cyclised to guanamine.

3. 5. 4. The effect of phenethybiguanide and 1,1-dimethylbiguanide.

In simultaneous studies on the same cells comparing phenethybiguanide with 1,1-dimethylbiguanide (metformin) (Figure 16A), it was shown that 1,1-dimethylbiguanide significantly decreased the activity to 38% of control at high concentration (10^{-4}M) compared to control (100%) ($p<0.01$; basal phosphorylase activity 32 $\pm$ 1.4 nmol/mg of protein/min). The cells were more sensitive to metformin, with greater effects occurring at all concentrations with metformin

Figure 15. Effect of various concentrations of (A) phenethylbiguanide (○) and phenethylguanamine (●), (B) phenylbiguanide (○) and phenylguanamine (●), (C) benzylbiguanide (○) and benzylguanamine (●) and insulin (▽) on glycogen phosphorylase a activity in hepatocytes isolated from insulin treated diabetic rat compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 5min exposure to the compounds and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All values are significant at $p<0.01$, except $*p<0.05$, † and # = not significant. Basal phosphorylase activity for control in graph A (32 ± 1.4 nmol/ mg of protein/ min), B (37 ± 6.5 nmol/ mg of protein/ min) and C (34 ± 3.5 nmol/ mg of protein/ min).


A
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PHOSPHORYLASE a ACTIVITY (% of control)
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*

compared to phenethyl biguanide ($p < 0.001$). The effect of metformin was similar to insulin at low (10^{-10}M) and high (10^{-5}M) concentrations.

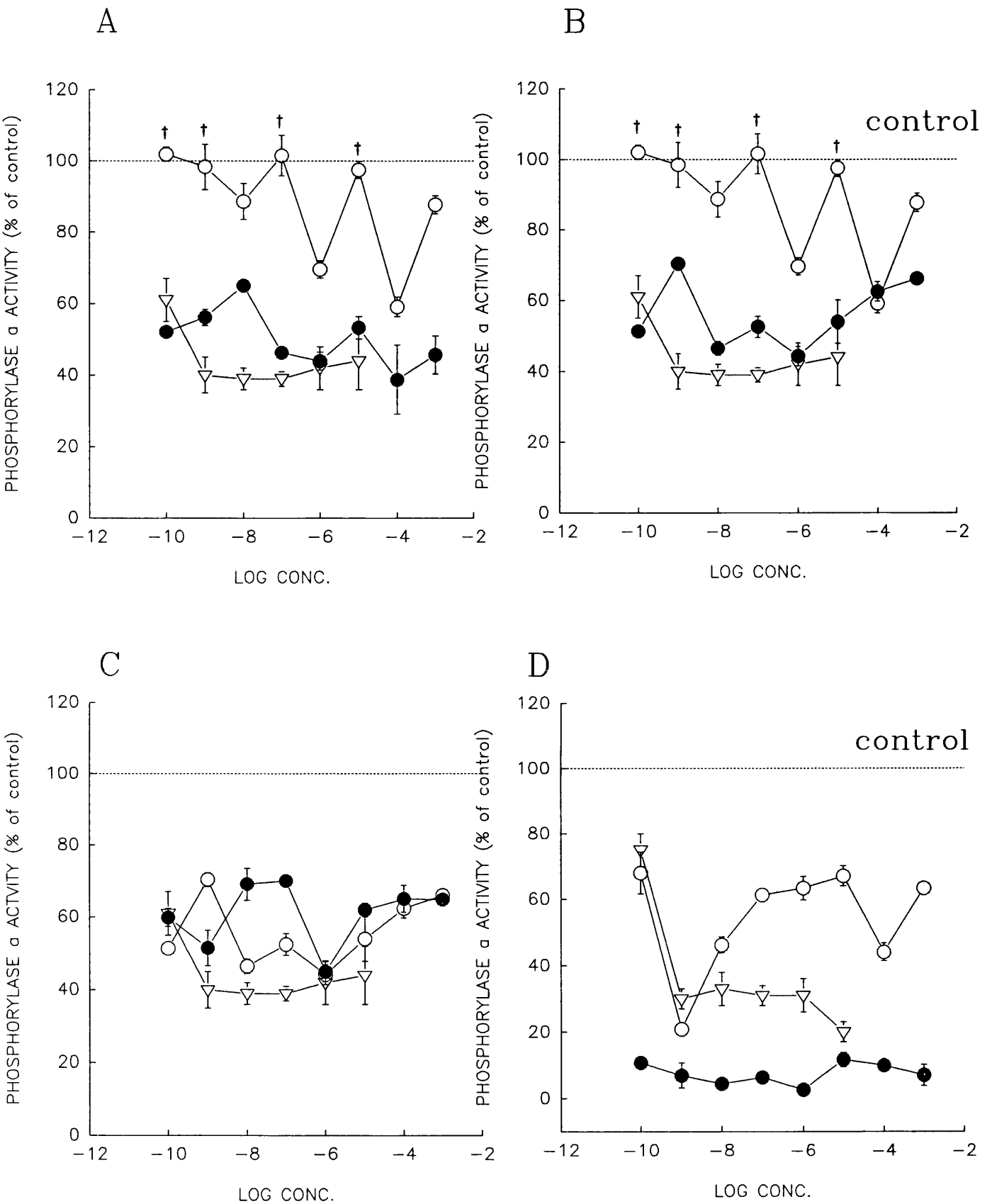
3. 5. 5. The effect of phenethylbiguanide and saccharin

On the basis of previous observations on the normal rat hepatocytes, we tried to compare the effect of saccharin (o-sulfobenzimide) and phenethylbiguanide. When the cells were treated with saccharin, it was seen that the activity decreased significantly at all concentrations compared to the control ($p<0.01$, basal phosphorylase activity 32 $\pm$ 1.4 nom/ mg of protein/ min) (Figure 16B). The result was slightly different in the case of phenethylbiguanide (phenformin) where it was observed that there was no difference in the activity at concentrations of 10^{-5}M, 10^{-7}M ,10^{-9}M and 10^{-10}M. The activity of phenformin was detectable only at the higher concentrations (10^{-3}M and 10^{-4}M) and at 10^{-6}M and 10^{-8}M. Saccharin at concentrations of 10^{-5}M and below decreased the activity significantly more than phenethylbiguanide ($p< 0.001$).

3. 5. 6. The effect of saccharin and diazoxide.

The addition of diazoxide (a hyperglycaemic drug which contains the same groups (amino and sulphur) as saccharin but in a 6-membered ring to hepatocytes isolated from insulin-treated diabetic rats (Figure 16C) showed that saccharin and diazoxide decrease the activity significantly compared to control ($p< 0.01$; basal phosphorylase activity 32 $\pm$ 1.4 nmol/ mg of protein/ min). The effect of diazoxide in hepatocytes isolated from insulin-treated diabetic rats is in contrast to the effect of the drug on the normal rat hepatocytes.

Figure 16. The effect of (A) phenethylbiguanide (phenformin) (O) and 1,1-dimethylbiguanide (metformin) (●), (B) phenethylbiguanide (O) and sulfobenzimid (saccharin) (●), (C) sulfobenzimide (saccharin) (O), 7 chloro-3 methyl-2H-1,2,4-benzothiadiazine 1,1-dioxid (diazoxide) (●), (D) 2,4,6-triamino-1,3,5-triazine (Melamine) (O), 3,5-diamino triazol (●) on phosphorylase a activity in hepatocytes isolated from insulin treated diabetic rats in comparison to control (100%). Hepatocytes were preincubated for 30 min in Krebs-Henseleit medium and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All values are significant at $p<0.01$, except † not significant. Basal activity of glycogen phosphorylase for control in graph A, B, C (32 ± 1.4 nmol/ mg of protein/ min), D (34 ± 5.5 nmol/ mg of protein/ min).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3. 5. 7. The effect of melamine and 3, 5-diaminotriazol.

The same effect was observed when melamine was used in normal hepatocytes (Figure 11D) and hepatocytes isolated from insulin-treated diabetic rats (Figure 16D). It completely inhibited the activity but this was found to be due to a decreased viability of the cells. 3,5-Diaminotriazole behaved, in its effects on the enzyme activity, as in normal rat hepatocytes by a decrease in the activity to 44% of control at high concentration (10^{-4}M) ($p< 0.01$; basal phosphorylase activity 34 $\pm$ 5.5 nmol/ mg of protein/ min). The maximum activity was observed at low concentrations (10^{-9}M) (20% to control).

3. 6. A change in the effect of biguanides, guanamines, miscellaneous compounds and insulin on glycogen phosphorylase a activity in hepatocytes from normal rats in different glucose concentrations.

In a previous study (refer to section 3.4) exposure of hepatocytes isolated from diabetic rats to biguanides, guanamines and insulin resulted in an increase in glycogen phosphorylase a activity. This series of experiments deals with the effect of biguanides, guanamines and insulin in normal rat hepatocytes after the cells were preincubated in Krebs-Henseleit medium containing 10mM and 30 mM glucose (Figure 18-23). Figure 17 shows that basal phosphorylase a activity was reduced by preincubation of hepatocytes in 30mM glucose to 37 $\pm$ 6 nmol/ mg of protein/ min) compared to basal phosphorylase a activity in hepatocytes from normal rats when pre incubated in 10mM glucose (59 $\pm$ 7 nmol/ mg of protein/ min).

For convenience, most presentations of results are divided into two graphs; the effect of biguanides, guanamines and insulin in the presence of 10mM glucose in

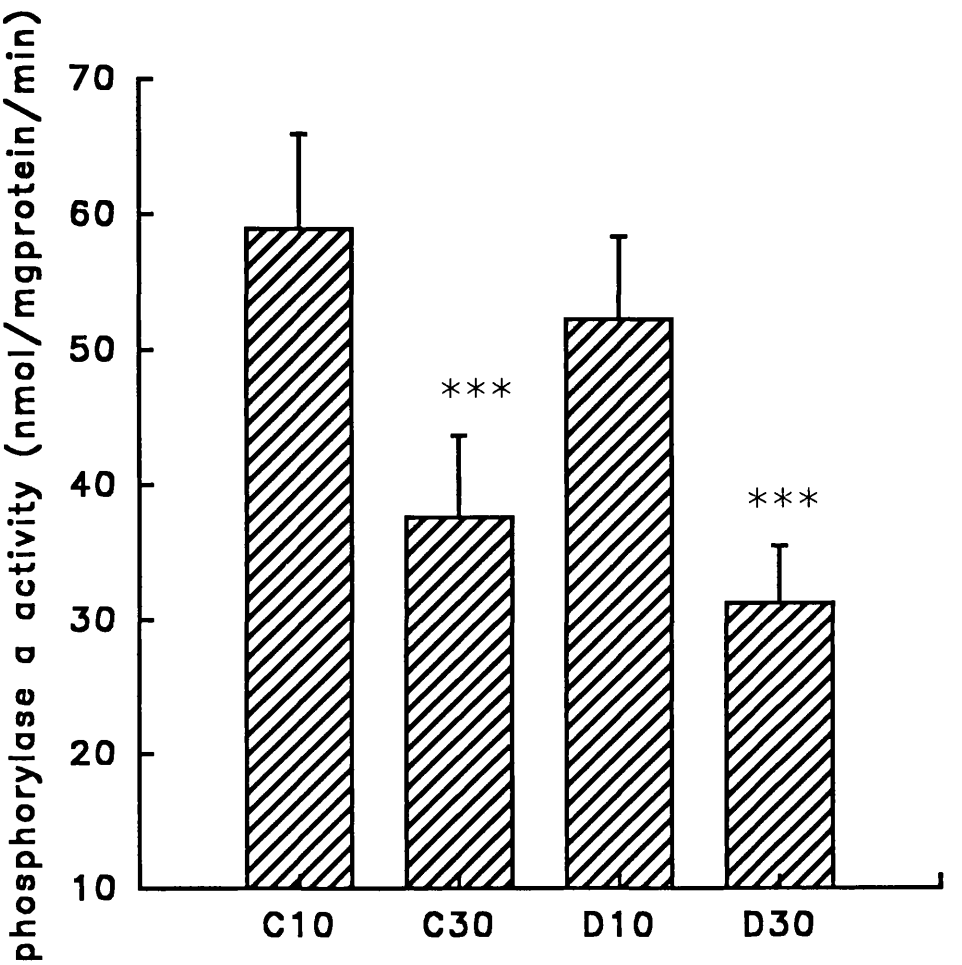


Figure 17. Glycogen phosphorylase a activity in control (10mM and 30mM glucose), streptozotocin-induced-diabetic rat (10mM and 30mM glucose). Values are the mean ± s.d. for eight animals. p values relative to the control values were obtained by ANOVA followed by Dunnet's test,***$p<0.01$. Basal phosphorylase activity for control in 10mM glucose (59 ± 7 nmol/ mg of protein/ min), in 30mM glucose (37 ± 6 nmol/ mg of protein/ min), basal phosphorylase activity for diabetic rat in 10mM (52 ± 6 nmol/ mg of protein/ min), in 30mM glucose (33 ± 4 nmol/ mg of protein/ min).

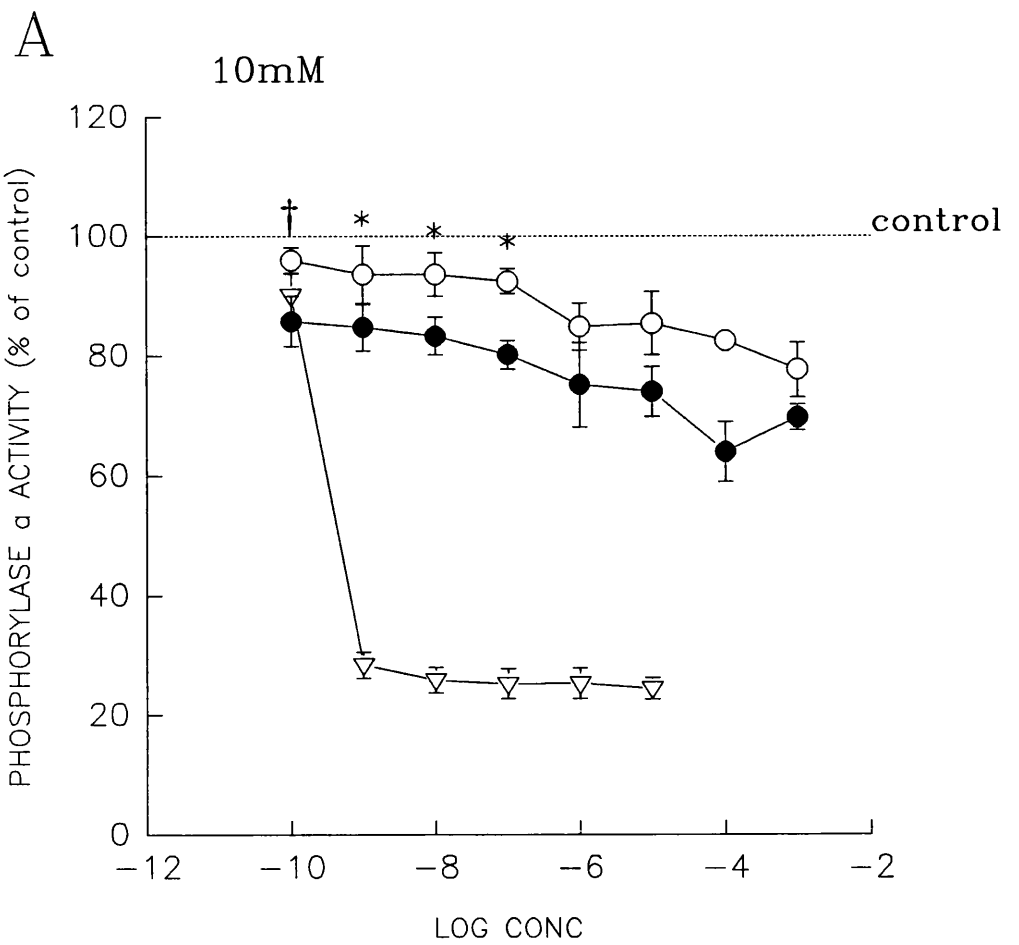
normal rat hepatocytes are presented in one graph and the effect of biguanides, guanamines and insulin in the presence of 30mM glucose in a second graph.

3. 6. 1. The effect of phenethylbiguanide and phenethylguanamine.

In Figure 18A phenethylbiguanide (phenformin) in the concentration range 10^{-9}M to 10^{-3}M, gave a significant decrease in the activity of glycogen phosphorylase a to 77% - 90% of control ($p< 0.05$; basal phosphorylase activity 51 $\pm$ 2.8 nmol/ mg of protein / min) in a dose-dependent manner in 10mM glucose as seen previously. There was no significant change in activity when phenethylbiguanide was used at a low concentration (10^{-10}M). The cyclization of phenethylbiguanide to phenethylguanamine increased the effectiveness of the compound in 10mM glucose causing a decrease in the activity of the enzyme in all concentrations from 10^{-10}M to 10^{-3}M in a dose - dependent way. The maximum activity (64% of control) occurred at the concentration of 10^{-4}M. Phenethylguanamine at concentrations 10^{-6}M to 10^{-4}M decreased the activity of the enzyme significantly more than phenethylbiguanide ($p< 0.01$). Similarly phosphorylase inactivation was observed at all insulin concentrations studied between 10^{-10}M and 10^{-5}M.

Preincubation of rat hepatocytes for 30 min with 30mM glucose resulted in a marked decrease in the basal activity of glycogen phosphorylase a to 13 $\pm$ 2.1 nmol/ mg of protein/ min (compared to 51 $\pm$ 3 nmol/ mg of protein/ min of control). Exposure of these rat hepatocytes to phenethylbiguanide for 5 min resulted in a significant increase in glycogen phosphorylase a (Figure 18B). This increase occurred at all phenethylbiguanide concentrations from 10^{-10}M to 10^{-3}M and gave a maximum effect at 10^{-4}M (196% of control). The cyclization of phenethylbiguanide to phenethyguanamine again increased the effectiveness of the compound exhibiting a dose-dependent effect on the activation of phosphorylase giving a maximum response of 270% of the control at 10^{-4}M. Phenethyguanamine increased enzyme activity more than phenethylbiguanide ($p< 0.01$). Insulin also increased the enzyme activity but in a

Figure 18. Dose response curves of glycogen phosphorylase to phenethylbiguanide (O), phenethylguanamine (●) and insulin (∇) in rat hepatocytes pre incubated for 30 min in (A) 10mM glucose (B) 30mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All value are signficant at $p < 0.01$ except *$p < 0.05$, † (not significant). basal phosphorylase activity for control in graph A (51 ± 3 nmol/ mg of protein/ min), B (13 ± 2nmol/ mg of protein/ min).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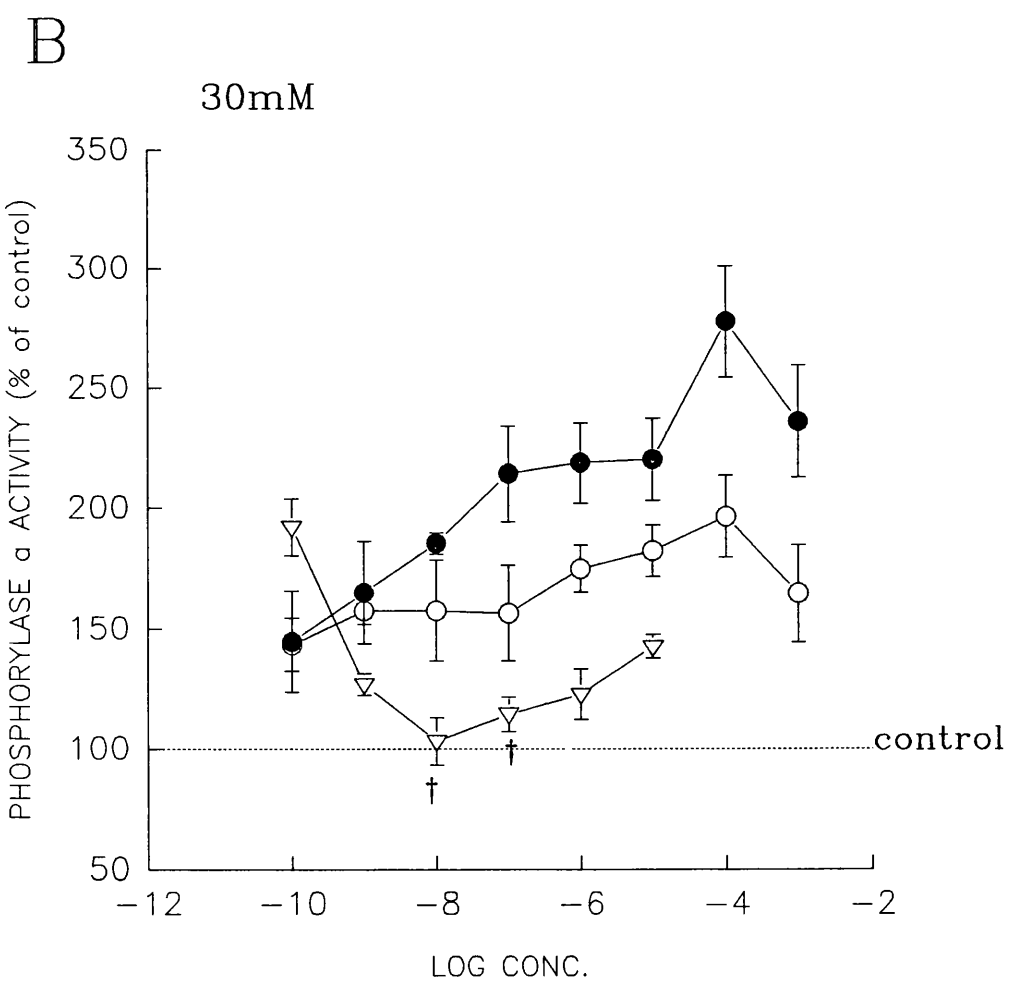
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PHOSPHORYLASE a ACTIVITY (% of control)
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LOG CONC.

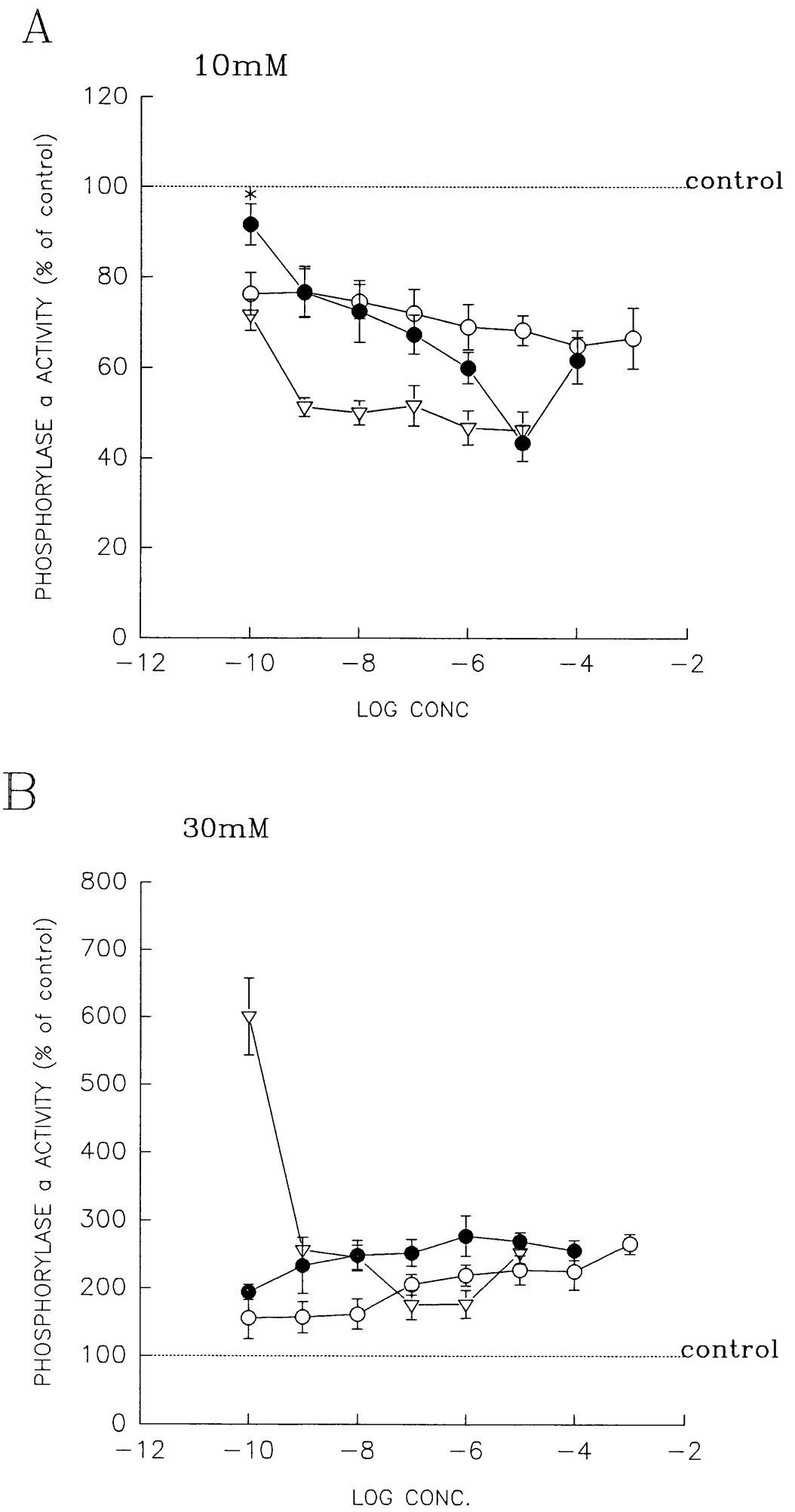
biphasic manner. The maximum activity was observed at low concentration 10^{-10}M with the effect disappearing at physiological concentration. Supraphysiological concentrations (greater than 10^{-7}M) also caused an increase in enzyme activity resulting in a U-shaped dose-response curve.

3. 6. 2. The effect of phenylbiguanide and phenylguanamine

As shown in figure 19A when hepatocytes were preincubated with 10mM glucose and then treated with phenylbiguanide, phenylguanamine or insulin, it was found that all concentrations of these drugs in the range 10^{-10}M to 10^{-5}M decreased the activity of glycogen phosphorylase a significantly compared to the control ($p< 0.01$ basal phosphorylase activity 34 $\pm$ 2.6 nmol/ mg of protein/ min). The exception is phenylguanamine at 10^{-10}M where $p<0.05$. Phenylguanamine exhibited a steeper dose response curve with maximum response achieved at 10^{-5}M and caused a greater decrease in enzyme activity compared to phenylbiguanide at concentrations of 10^{-6}M and 10^{-5}M ($p< 0.01$). A comparison of both compounds with insulin showed that insulin had a greater effect on enzyme activity (giving an inhibition to 46% of control at 10^{-9}M ($p< 0.01$)). This was significantly greater than the effect of phenylbiguanide or phenylguanamine ($p< 0.001$).

When hepatocytes were preincubated in 30mM glucose and exposed to phenylbiguanide and phenylguanamine, the activity of glycogen phosphorylase a was increased as shown in Figure 19B. Phenylbiguanide significantly increased the activity of glycogen phosphorylase a to 155% - 265% of control ($p< 0.01$; basal phosphorylase a activity 8 $\pm$ 1 nmol/ mg of protein/ min). in a dose - dependent manner with the maximum response at 10^{-3}M. A similar dose - response effect was seen for phenylguanamine, at concentrations of 10^{-10}M to 10^{-6}M reaching to about 194% to 277% of the control with a maximum increase of enzyme activity occurring at 10^{-6}M and a subsequent fall in enzyme activity at the higher concentrations. Phenylguanamine

Figure 19. Effect of various concentrations of phenylbiguanide (○), phenylguanamine (●) and insulin (▽) on glycogen phosphorylase activity in normal rat hepatocytes to control (100%). The cells pre incubated for 30 min in (A) 10mM glucose (B) 30mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All value were significant at $p < 0.01$ except $*p < 0.05$. basal phosphorylase activity for control in graph A (34 ± 3 nmol/ mg of protein/ min), B (8 ± 1 nmol/ mg of protein/ min).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LOG CONC.

increased the enzyme activity significantly more at all concentrations ($p< 0.05$) than phenylbiguanide.

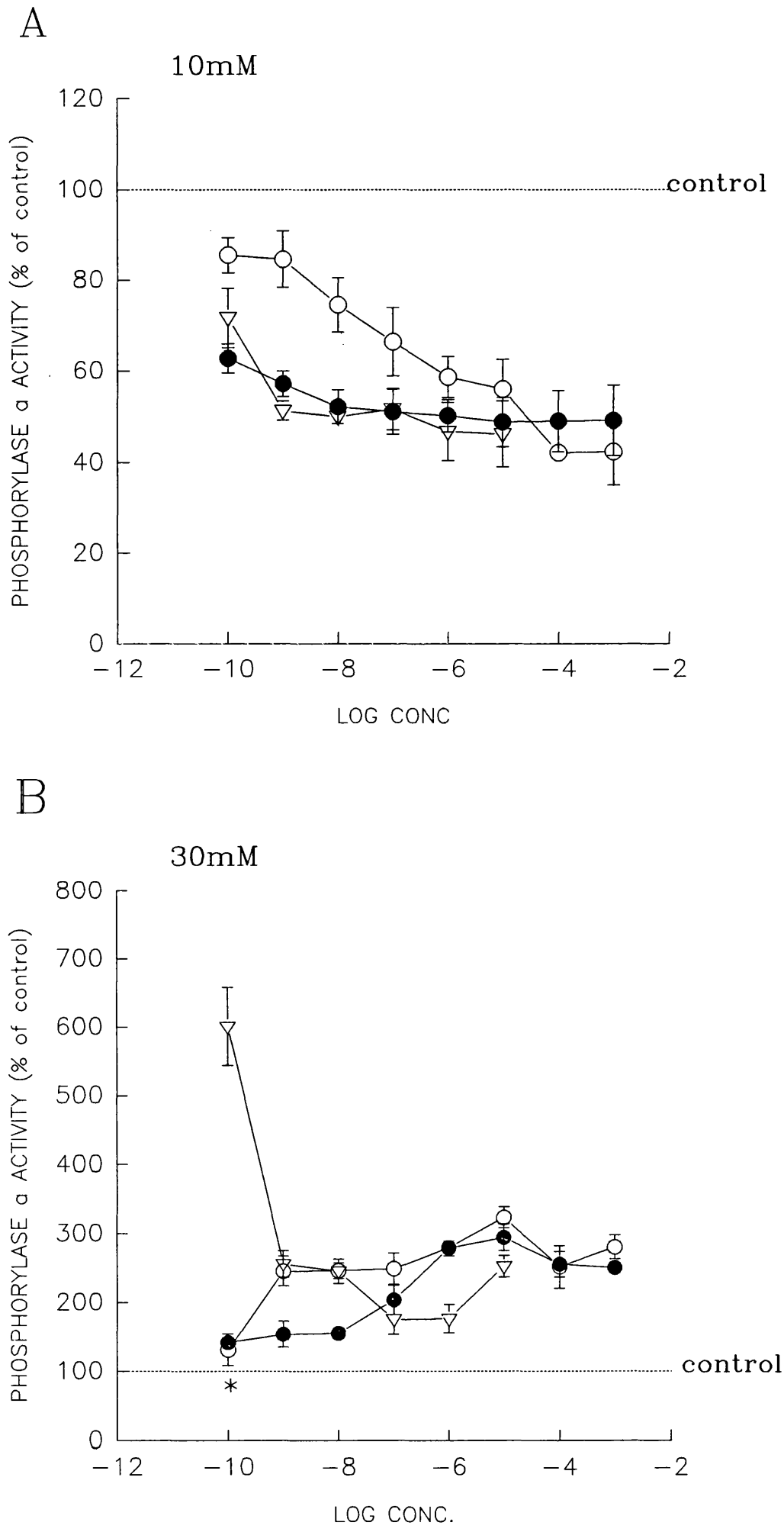
As shown in Figure 19B when cells were exposed to 30mM glucose followed by different concentrations of insulin, the enzyme exhibited a U-shaped-dose response curve with a significant increase in the enzyme activity at concentrations of 10^{-10}M to 10^{-5}M. Insulin at 10^{-10}M gave the maximum response reaching 600% of control ($p< 0.01$; basal phosphorylase activity 8 $\pm$ 1 nmol/ mg of protein/ min). Enzyme activity declined sharply above this concentration but remained significantly above control. At the physiological concentration 10^{-9}M activity was 256% of control and at 10^{-5}M activity was 252% of control.

3. 6. 3. The effect of benzylbiguanide and benzylguanamine.

In similar experiments, the actions of benzylbiguanide, benzylguanamine and insulin on glycogen phosphorylase a was studied (Figure 20). In hepatocytes from normal animals cultured in 10mM glucose, benzylbiguanide at concentrations in the range 10^{-10}M to 10^{-3}M decreased the enzyme activity in a dose dependent manner compared to control ($p< 0.01$; basal phosphorylase activity 34 $\pm$ 2 nmol/ mg of protein/ min) with a maximum activity observed at 10^{-4}M and 10^{-3}M (Figure 20A). The cyclization of benzylbiguanide to benzylguanamine improved activity compared to the control ($p< 0.01$) at all concentrations used up to 10^{-6}M. At concentrations up to 10^{-7}M the enzyme activity decreased significantly ($p< 0.001$) more than with benzylbiguanide. As shown in Figure 20A, insulin caused a dose - dependent decrease in enzyme activity and there was no significant difference in the effect compared to benzylguanamine.

In contrast to hepatocytes from normal rats preincubated in 10mM which exhibit a dose - dependent decrease in the enzyme activity with all compounds added preincubation with 30mM glucose led to a dose - dependent increase in enzyme activity with all compounds tested (Figure 20B). A similar dose - dependent effect for

Figure 20. Dose-response effect of glycogen phosphorylase a activity to benzylbiguanide (○), benzylguanamine (●) and insulin (▽) after normal rat hepatocytes were pre incubated for 30 min in (A) 10 mM glucose (B) 30 mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal phosphorylase activity for control in graph A (34 ± 3 nmol/ mg of protein/ min), B (8 ± 1 nmol/ mg of protein/ min). All values were significant at $p<0.01$.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PHOSPHORYLASE a ACTIVITY (% of control)
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LOG CONC.

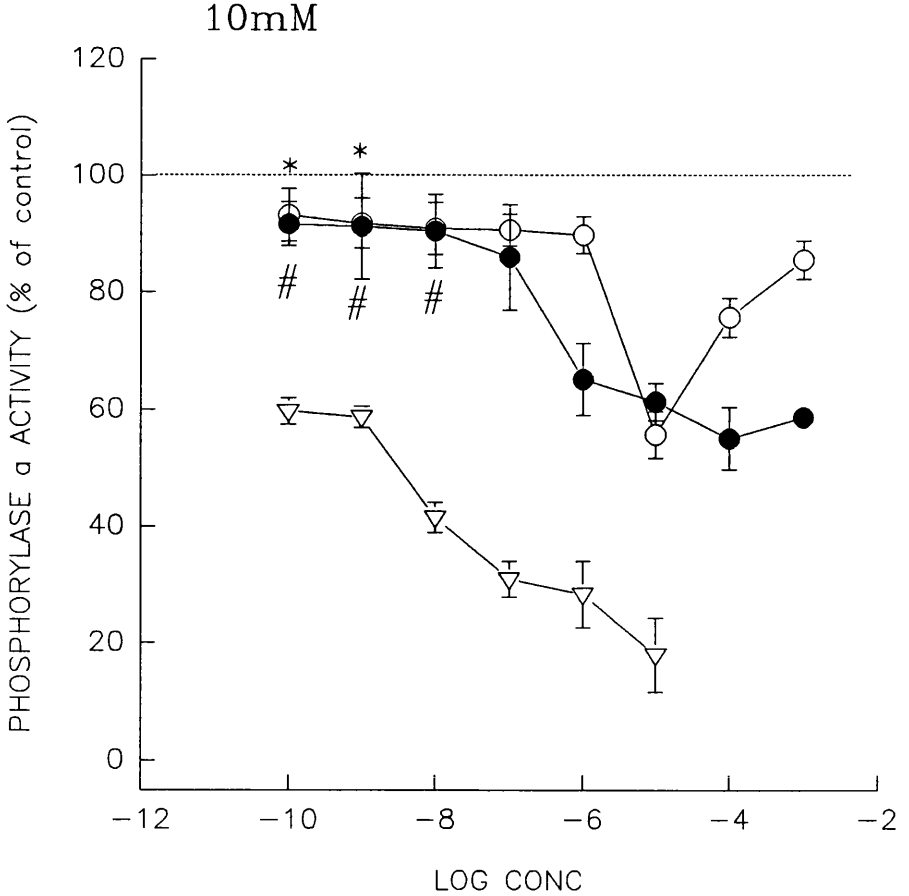
benzylbiguanide to that observed for phenyl and phenethylbiguanide was seen. At concentrations from 10^{-10}M to 10^{-5}M, benzylbiguanide elicited a dose - dependent increase in the activity of the enzyme reaching to 324% of the control at 10^{-5}M. At higher concentrations the effect of benzylbiguanide was reduced, although it was still higher than control at 10^{-3}M. Benzylguanamine significantly increased enzyme activity at all concentrations ($p< 0.01$; basal phosphorylase activity 8 $\pm$ 1 nmol of ^{14}C-glucose 1 phosphate/ mg of protein/ min) with the maximum effect observed at 10^{-5}M. At higher concentrations 10^{-4}M and 10^{-3}M the effect of benzylguanamine was reduced despite being significantly greater than their respective control. Figure 20B, shows the cyclization of benzylbiguanide to benzylguanamine did not improve overall the activity on glycogen phosphorylase, indeed there was less of an effect at the lower concentrations of 10^{-9} and 10^{-8}M). The same effect for insulin is shown in Figure 19B as was observed in Figure 20B with a U-shaped dose- response curve.

3. 6. 4. The effect of phenethylbiguanide and 1,1-dimethylbiguanide.

Both phenethylbiguanide (phenformin) and 1,1-dimethylbiguanide (metformin) were examined with normal rat hepatocytes cultured in 10mM and 30mM glucose to see which R- group has more effect and how change in the activity is affected by glucose concentration. In 10mM glucose, phenethylbiguanide (phenformin) decreased the activity significantly compared to control ($p<0.01$; basal phosphorylase activity 76 $\pm$ 5 nmol /mg of protein/ min) at all concentrations, as shown Figure 21A. Furthermore 1,1-dimethylbiguanide significantly decreased the activity to 55% of control ($p< 0.01$). At some concentrations (10^{-6}M, 10^{-4}M and 10^{-3}M) dimethylbiguanide decreased the enzyme activity significantly more than phenethylbiguanide ($p<0.001$). Insulin decreased enzyme activity significantly at all

Figure 21. Effect of various concentrations of phenethylbiguanide (phenformin) (○), 1,1-dimethylbiguanide (metformin) (●) and insulin (▽) on glycogen phosphorylase activity in normal rat hepatocytes compared to control (100%). The cells were pre incubated for 30 min in (A) 10 mM glucose (B) 30 mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal phosphorylase activity for control in graph A (76 ± 5 nmol/ mg of protein/ min) B (57 ± 4 nmol/ mg of protien/ min). All values are significant at $p<0.01$ except * $p<0.05$ and † (not significant).

A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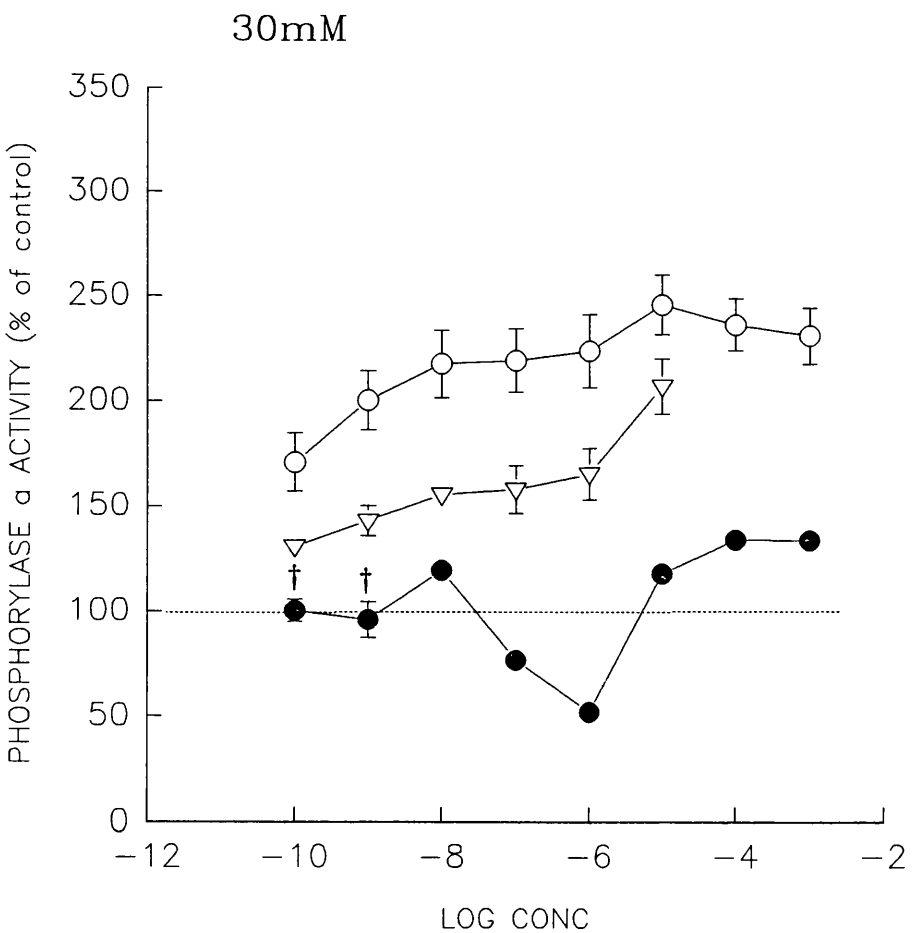
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concentrations compared to control ($p< 0.01$) and compared to phenethylbiguanide and phenethylguanamine ($p< 0.001$).

In simultaneous studies with the same cells, by changing the glucose concentration from 10mM to 30mM, a comparison of the effects of phenethylbiguanide (phenformin) with 1,1-dimethylbiguanide (metformin) was made. It showed that, in 30mM glucose, phenethylbiguanide increased the enzyme activity significantly at all concentrations (Figure 21B) ($p<0.01$; basal phosphorylase activity 57 $\pm$ 4 nmol/ mg of protein/ min). It can be further seen from Figure 21B that 1,1dimethylbiguanide increased the enzyme activity to a lesser extent than phenethylbiguanide. Indeed, only at concentrations of 10^{-8}M, 10^{-5}M and 10^{-3}M was the enzyme activity increased significantly ($p< 0.01$). At concentrations of 10^{-6}M and 10^{-7}M the enzyme activity was reduced significantly below control. Insulin caused a dose - dependent increase in enzymes activity with a maximum response seen at 10^{-5}M. In comparison with phenethylbiguanide and 1,1-dimethylbiguanide, it was observed that insulin increased the activity significantly more ($p< 0.001$) than 1,1dimethylbiguanide, but less than phenethylbiguanide ($p< 0.001$).

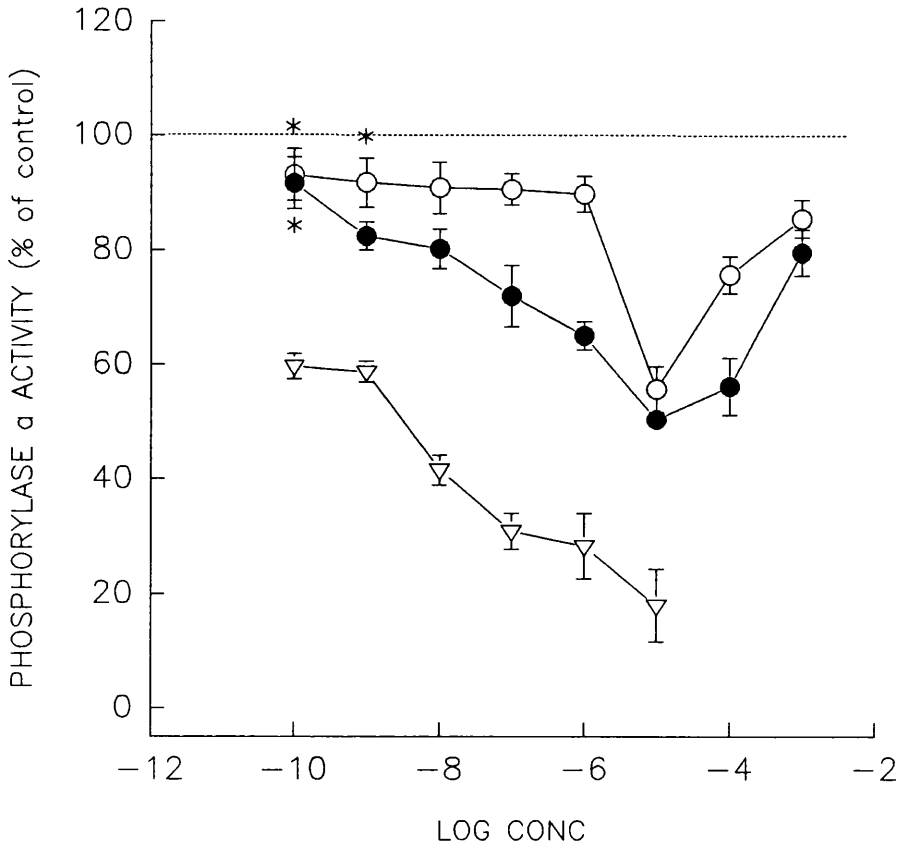
3. 6. 5. The effect of phenethylbiguanide and saccharin.

It can be further seen from Figure 22A, when phenethylbiguanide was compared with saccharin (o-sulfobenzimide) in 10mM glucose, that saccharin decreases the enzyme activity significantly in all concentrations compared to the control ($p<0.01$) (basal phosphorylase activity 76 $\pm$ 5 nmol/ mg of protein / min). There was no difference in the effect of the two compounds at low concentration (10^{-10}M), but saccharin decreased the enzyme activity significantly more at concentrations of 10^{-9}M and above.

Also in this experiment, the effects of the same compounds on normal rat hepatocytes cultured in 30mM glucose was examined. Figure 22B shows that there is

Figure 22. Dose response curves of glycogen phosphorylase phenethylbiguanide (phenformin) (○), saccharin (o-sulfobenzimide) (●) and insulin (▽) in normal rat hepatocytes pre incubated for 30 min in (A) 10 mM glucose (B) 30 mM glucose and the effect of 5 min expousure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test, compared to basal phosphorylase activity (100%). Basal phosphorylase activity for control in graph A (79 ± 5 nmol/ mg of protein/ min), B (57 ± 4 nmol/ mg of protein / min). All values are significant at $p < 0.01$, except * $p < 0.05$, † (not significant).

A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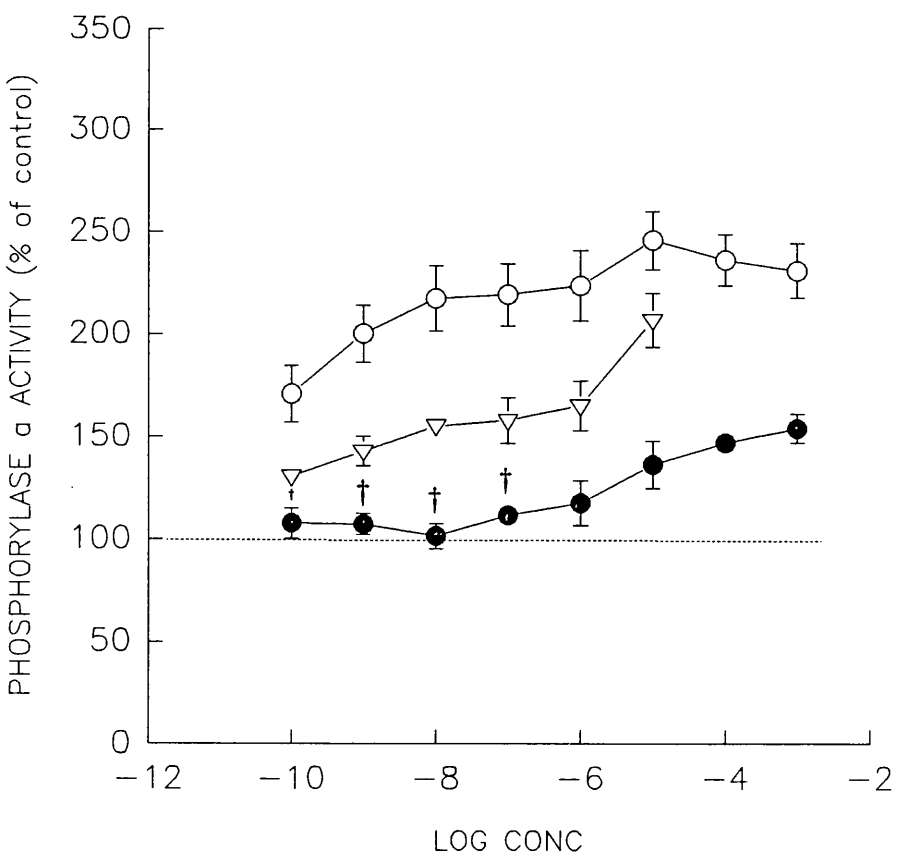
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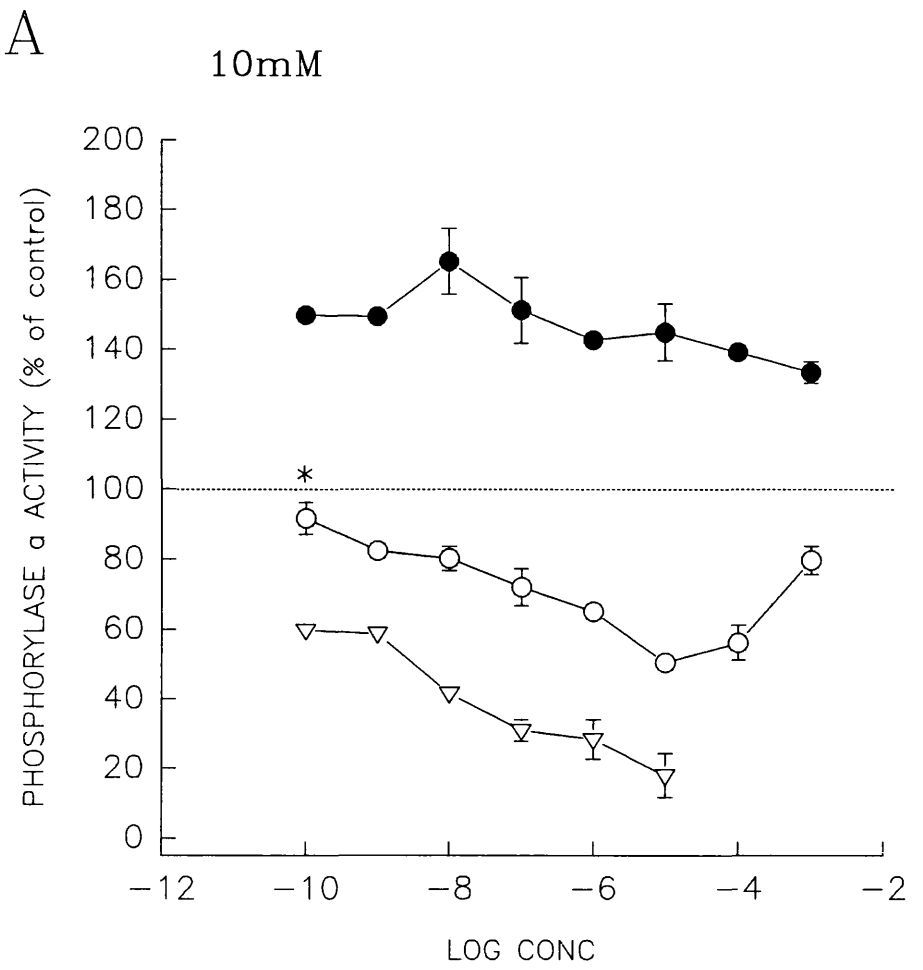
no change in enzyme activity when saccharin was added at low concentrations (10^{-10}M to 10^{-7}M) but at higher concentrations (10^{-6}M to 10^{-3}M) saccharin increased enzyme activity significantly ($p< 0.01$; basal phosphorylase activity 57 $\pm$ 4 nmol/ mg of protein/ min). Phenethylbiguanide increased the activity significantly at all concentrations ($p<0.01$) and was significantly better than saccharin ($p<0.001$). Phenethylbiguanide also increased the enzyme activity significantly better at all concentrations compared to insulin ($p<0.001$).

3. 6. 6. The effect of saccharin and diazoxide

In the comparison between saccharin and diazoxide, hepatocytes from normal rats preincubated in 10mM glucose were treated with diazoxide to compare with hepatocytes treated with saccharin. Diazoxide increased the enzyme activity significantly in all concentrations compared to the control (Figure 23A) ($p<0.01$; basal enzyme activity 76 $\pm$ 5 nmol/ mg of protein/ min). The maximum effect of diazoxide was seen at 10^{-8}M. Attenuation of diazoxide's effect could be seen when the concentration was increased above 10^{-7}M. Saccharin, as previously mentioned, caused a significant decrease in enzyme activity. When the cells were pre incubated in 30mM glucose and exposed to diazoxide, there was a significant increase in the enzyme activity compared to control ($p<0.01$ basal phosphorylase activity 57 $\pm$ 4 nmol/ mg of protein/ min) (Figure 23B). Saccharin also caused a significant increase in enzyme activity at concentrations higher than 10^{-6}M. Diazoxide was more effective than either insulin or saccharin in increasing enzyme activity significantly ($p< 0.01$).

Figure 23. The effect of saccharin (o-sulfobenzimid) (○), diazoxide (7 chloro-3-methyl -2H-1,2,4- benzothiadiazine) (●) and insulin (∇) on phosphorylase a activity in normal rat hepatocytes were pre incubated for 30 min in (A) 10mM glucose (B) 30mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6) analysed by ANOVA followed by Dunnett's test. All values are significant at $p<0.01$, except † (not significant). basal activity of glycogen phosphorylase for control in graph A (76 ± 5 nmol/ mg of protein/ min), B (57 ± 4 nmol/ mg of protien/ min).


A
10mM
PHOSPHORYLASE a ACTIVITY (% of control)
200
180
160
140
120
100
80
60
40
20
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*
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−10
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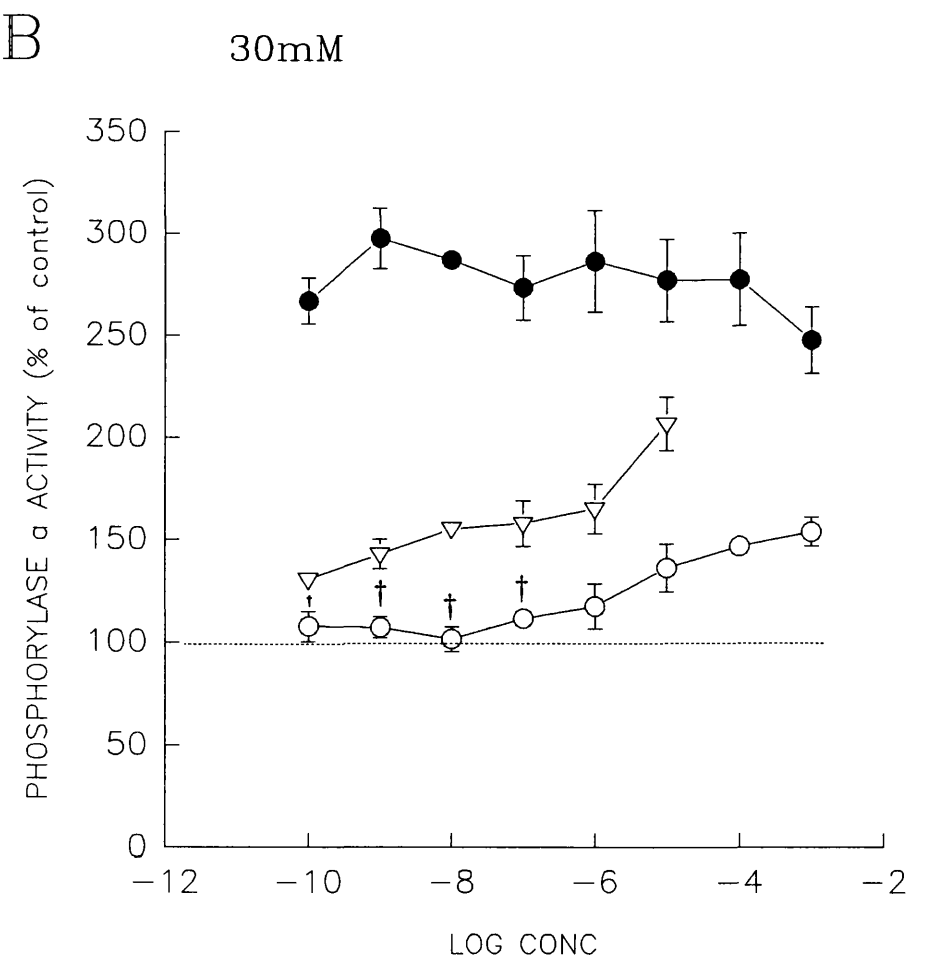
LOG CONC


B
30mM
PHOSPHORYLASE a ACTIVITY (% of control)
350
300
250
200
150
100
50
0
†
−12
−10
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−6
−4
−2
LOG CONC

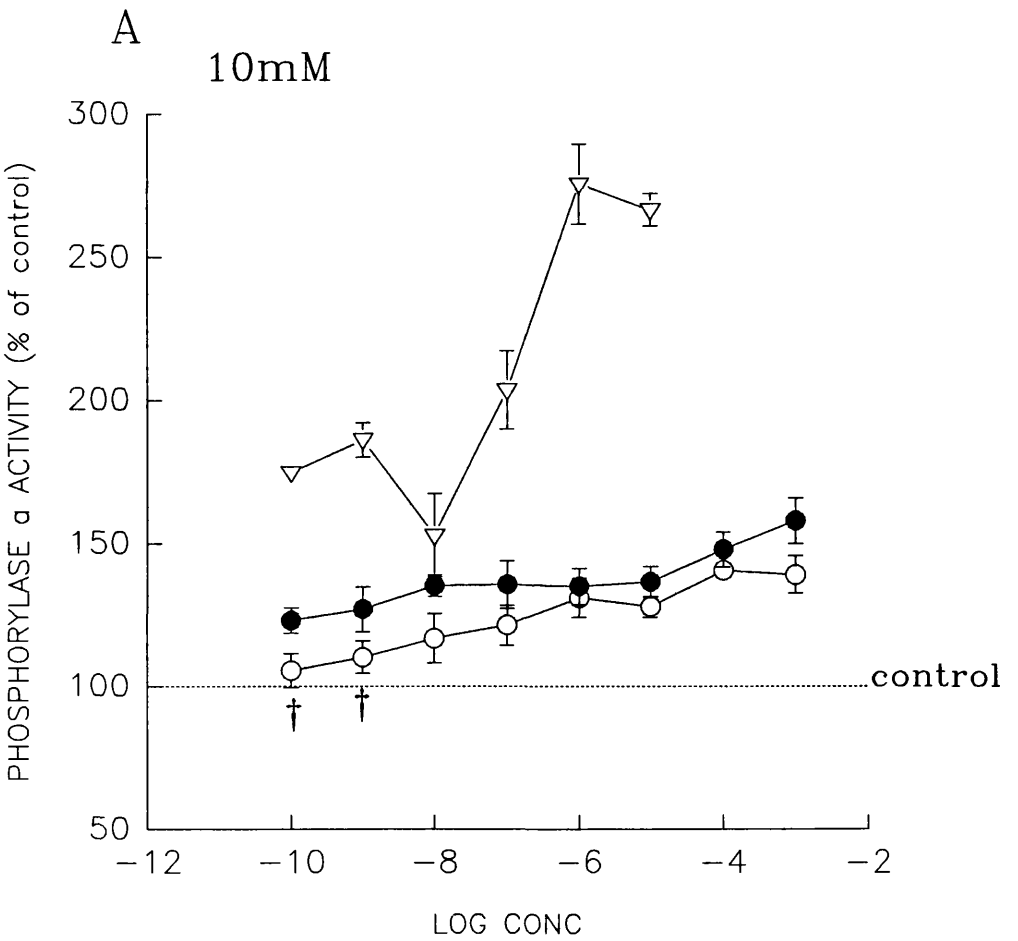
3. 7. Effect of biguanides, guanamines and insulin on the activity of glycogen phosphorylase a in hepatocytes isolated from STZ diabetic rats in different glucose concentrations.

The actions of biguanides, guanamines and insulin on glycogen phosphorylase a were further studied by analysing their effects in hepatocytes isolated from diabetic rats in 10 and 30mM glucose. Figure 17 shows that basal phosphorylase a activity was reduced to 31 $\pm$ 4 nmol/ mg of protein/ min when the hepatocytes isolated from diabetic rats were pre incubated with 30mM glucose compared with the activity when hepatocytes were pre incubated with 10mM glucose (52$\pm$6 nmol/ mg of protein/ min).

3. 7. 1. The effect of phenethylbiguanide and phenethylguanamine.

Figure 24A depicts the dose dependent effect of phenethylbiguanide, phenethylguanamine and insulin on the glycogen phosphorylase a in diabetic hepatocytes incubated in 10mM glucose. Phenethylbiguanide in the concentrations range 10^{-8}M to 10^{-3}M increased the activity significantly to 140% of control at a concentration of 10^{-4}M ($p<0.01$; basal phosphorylase activity 52 $\pm$ 6 nmol/ mg of protein/ min) There was no significant change in the activity when phenethylbiguanide was used at low concentration (10^{-10}M to 10^{-9}M). The cyclization of phenethylbiguanide to phenethylguanamine increased the effectiveness of the compound causing dose - dependent increase in all concentrations from 10^{-10}M to 10^{-3}M. Maximum effect was seen at a concentration of 10^{-3}M (158% of control). Phenethylguanamine at the concentrations of 10^{-8}M, 10^{-5}M and 10^{-7}M increased the activity of the enzyme significantly more than phenethylbiguanide ($p<0.01$ and $p< 0.05$ respectively). Insulin stimulated glycogen phosphorylase a in a dose-dependent manner with maximal effect at 10^{-6}M (275% of control) ($p< 0.01$). Insulin increased the activity significantly in the concentration range 10^{-8}M to 10^{-5}M ($p<0.001$) compared to phenethylbiguanide and phenethylguanamine.

Figure 24. Dose response curves of glycogen phosphorylase to phenethylbiguanide (O), phenethylguanamine (●) and insulin (∇) in diabetic rat hepatocytes pre incubated for 30 min in (A) 10mM glucose (B) 30mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All value are significant at $p < 0.01$, except † (not significant). Basal phosphorylase activity for control in graph A (52 ± 6 nmol/ mg of protein/ min), B (33 ± 4 nmol/ mg of protein/ min).


A
10mM
300
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200
150
100
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PHOSPHORYLASE a ACTIVITY (% of control)
control
−12
−10
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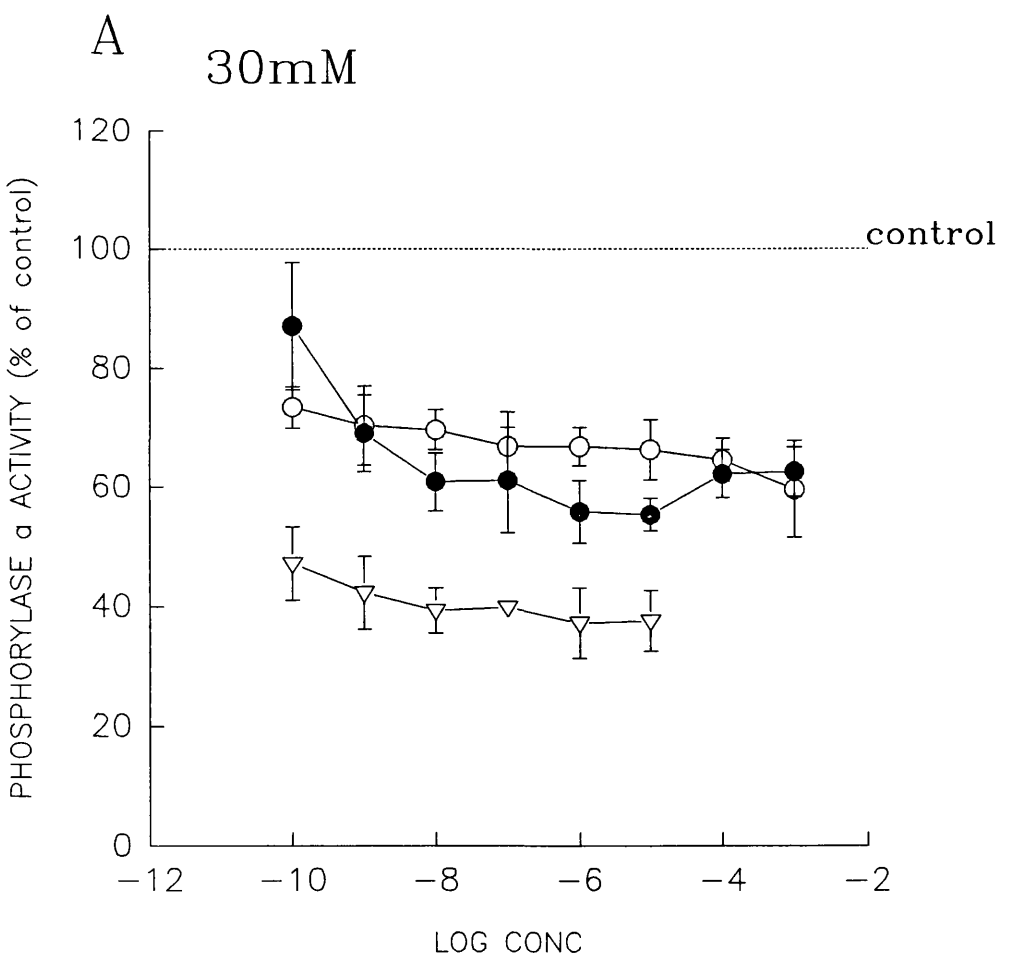
LOG CONC


A
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120
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80
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0
PHOSPHORYLASE a ACTIVITY (% of control)
control
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LOG CONC

Preincubation of the hepatocytes for 30 min with 30mM glucose resulted in a marked decrease in basal activity of glycogen phosphorylase a (Figure 17). Exposure of these hepatocytes to phenethylbiguanide, phenethylguanamine and insulin for 5 min resulted in a further inactivation of the enzyme. Both phenethylbiguanide and phenethylguanamine in the concentration range 10^{-10}M to 10^{-3}M caused a dose dependent decrease in enzyme activity (Figure 24B) ($p< 0.01$; basal phosphorylase activity 31 $\pm$ 4 nmol/ mg of protein/ min). In this experiment, using the same cells, insulin was more effective than phenethylbiguanide or phenethylguanamine ($p< 0.001$).

3. 7. 2. The effect of phenylbiguanide and phenylguanamine.

Changing the phenethyl group to a phenyl group was examined on the same set of cells isolated from diabetic rats in 10mM glucose. As shown in Figure 25A phenylbiguanide stimulated the activity of glycogen phosphorylase in a dose dependent way at all concentrations used ($p<0.01$; control value = 52 $\pm$ 6 nmol/ mg of protein/ min). The cyclization of phenylbiguanide to phenylguanamine did not improve the activity, indeed less stimulation of glycogen phosphorylase a was seen, although statistically significant above control ($p< 0.01$). The same effect of insulin shown in Figure 24A was observed in Figure 25A with a dose-dependent increase. As shown in Figure 25B, when hepatocytes were preincubated with 30mM glucose and then treated with phenethybiguanide and phenethylguanamine, it is seen that with concentrations between 10^{-8}M to 10^{-3}M, the drugs decreased the activity of glycogen phosphorylase significantly compared to the control ($p< 0.01$; basal phosphorylase activity 31 $\pm$ 4 nmol/ mg of protein/ min). Phenethyguanamine did not alter activity as much as phenylbiguanide or insulin.

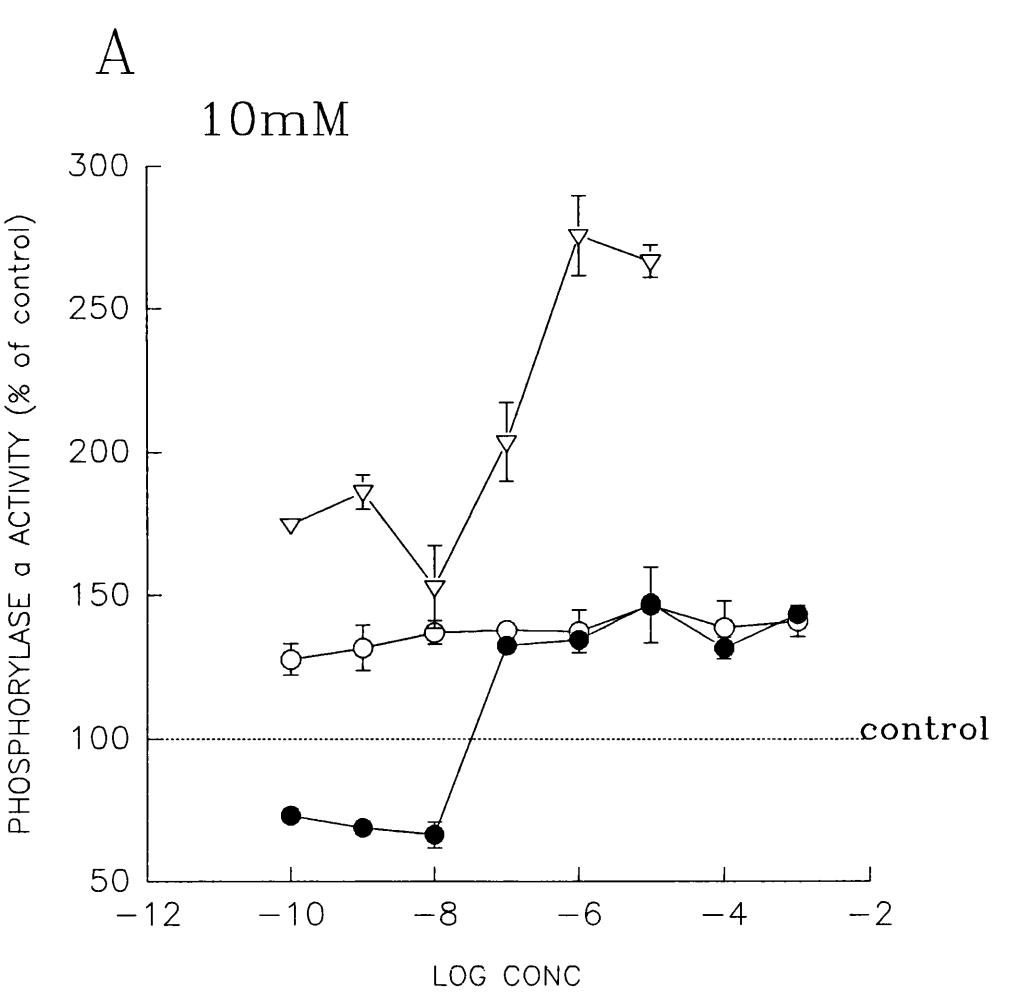
3. 7. 3. The effect of benzylbiguanide and benzylguanamine.

In the same set of cells isolated from diabetic rats, the action of benzylbiguanide, benzylguanamine and insulin on glycogen phosphorylase was studied (Figure 26A). In hepatocytes from diabetic animals preincubated in 10mM glucose, benzylbiguanide, at concentrations of 10^{-10}M to 10^{-3}M, increased the enzyme activity in a dose-dependent manner compared to control ($p< 0.01$; basal phosphorylase activity 52 $\pm$ 6 nmol/ mg of protein/ min) with a maximum effect observed at 10^{-5}M. The cyclization of benzylbiguanide to benzylguanamine gave a compound which increased activity significantly only at concentrations above 10^{-7}M ($p<0.01$). Indeed, at 10^{-10} to 10^{-8}M, a significant decrease in activity of the enzyme was seen. In contrast to hepatocytes from diabetic rats pre incubated in 10mM which exhibited a dose-dependent increase in the enzyme activity, preincubation of the same cells with 30mM glucose caused a dose-dependent decrease in enzyme activity (Figure 26B). A similar dose-dependent effect for benzylbiguanide to that observed for phenyl and phenethybiguanide (Figure 24B and 25B) was seen. At concentrations of 10^{-10}M to 10^{-3}M benzylbiguanide elicited a dose-dependent decrease in the activity of the enzyme reaching to 44% of control at high concentration ($p< 0.01$; basal phosphorylase activity 31 $\pm$ 4 nmol/ mg of protein/ min). The cyclization of benzylbiguanide to benzylguanamine increased the effect on the enzyme activity.

3. 8. ANDROST-4-ENE-3,17-DIONE METABOLISM IN HEPATOCYTES ISOLATED FROM NORMAL AND STREPTOZOTOCIN DIABETIC RATS IN DIFFERENT GLUCOSE CONCENTRATIONS.

A further aim of this study was to investigate the effect of biguanides and guanamines on androst-4-ene-3,17-dione metabolism, which has previously been shown to be insulin-dependent (Hussin & Skett, 1988). The assay for the metabolism

Figure 26. Effect of various concentrations of benzylbiguanide (O), benzylguanamine (●) and insulin (∇) on glycogen phosphorylase a activity in diabetic rat hepatocytes to control (100%). The cells pre incubated for 30 min in (A) 10mM glucose (B) 30mM glucose and the effect of 5 min exposure to the compounds and hormone on the enzyme activity measured. Value are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. All values significant at $p < 0.01$. Basal phosphorylase activity for control in graph A (34 ± 3 nmol/ mg of protein/ min), B (8 ± 1 nmol/ mg of protein/ min).


A
10mM
300
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control
PHOSPHORYLASE a ACTIVITY (% of control)
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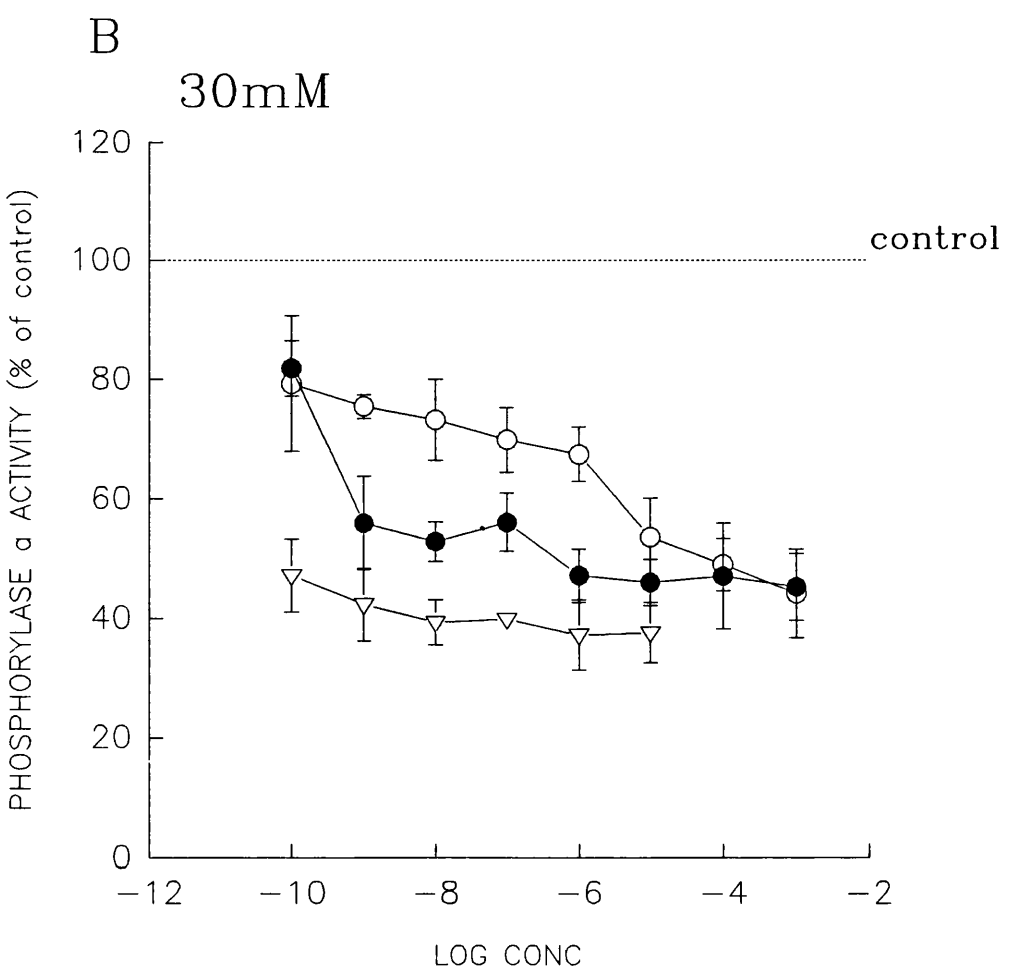
LOG CONC


B
30mM
120
100
80
60
40
20
0
control
PHOSPHORYLASE a ACTIVITY (% of control)
−12
−10
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LOG CONC

of androst-4-ene-3,17-dione enables the measurement of the activities of the enzymes 7α-, 6β- and 16α-hydroxylase, 17-oxosteroid oxidoreductase (17-OHSD) and 5α-reductase. Hepatocytes from normal and streptozotocin diabetic rats were isolated and preincubated in different glucose concentrations (10mM and 30mM) to simulate normal and diabetic conditions. Such conditions have been shown earlier to influence the activity of another insulin-dependent enzyme, glycogen phosphorylase.

3.8.1. 7α- hydroxylase activity

As shown in Figure 27 there was no significant difference in the activity when normal rat hepatocytes were preincubated in 30mM glucose (basal 7α- hydroxylase activity 22 ± 3 pmol/ mg of protein/ min) compared to cells which were preincubated in 10mM glucose (basal 7α- hydroxylase activity 22 ± 3 pmol/ mg of protein/ min). In diabetic hepatocytes the activity increased significantly to 94 ± 19 pmol/ mg of protein/ min in 10mM glucose compared to normal rat hepatocytes preincubated in 10mM and 30mM glucose ($p < 0.001$). The activity was increased significantly by preincubation of the cells in 30mM glucose ($p < 0.05$; basal 7α- hydroxylase activity 118 ± 24 pmol/ mg of protein/ min) compared to diabetic hepatocytes preincubated in 10mM glucose.

3. 8. 2. 6β- hydroxylase activity.

Similar results were obtained for the 6ß-hydroxylase as were seen above for the 7α-hydroxylase (Figure 28). Again there was no change in activity with changes in the concentration of glucose in normal rat hepatocytes whereas in diabetic hepatocytes activity was increased significantly to 85 ± 17 pmol/ mg of protein/ min in 10mM glucose compared to normal rat hepatocytes in 10mM glucose and 30mM glucose ($p < 0.001$). When the cells were preincubated in 30mM glucose the activity increased to

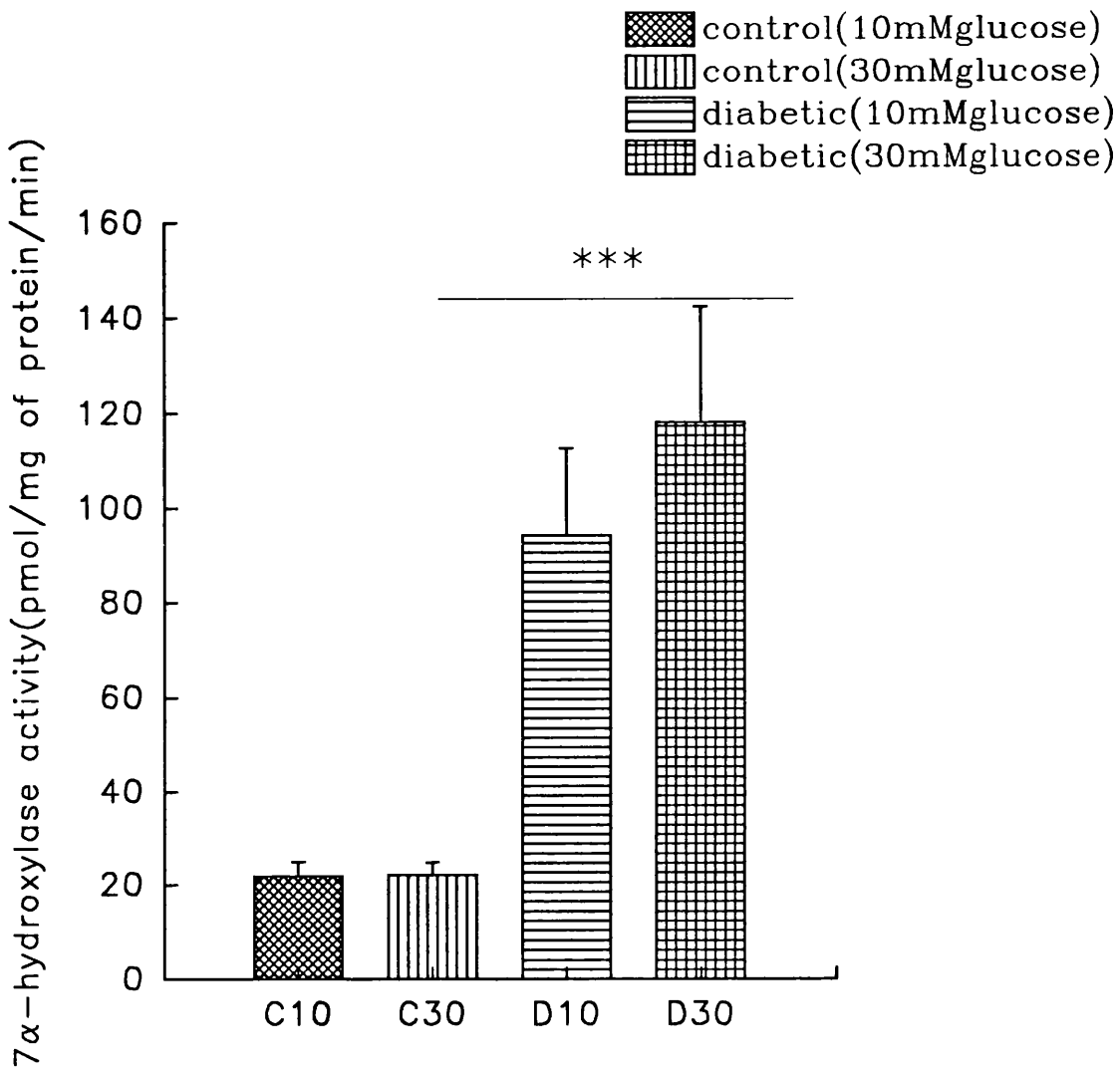


Figure 27. 7α-hydroxylase activity in control and streptozotocin- induced diabetic rat hepatocytes (in 10mM glucose and 30mM glucose). Values are the mean $\pm$ s.d for eight animals, p values relative to control values were obtained by ANOVA followed by Dunnet's test, *** $p < 0.001$.

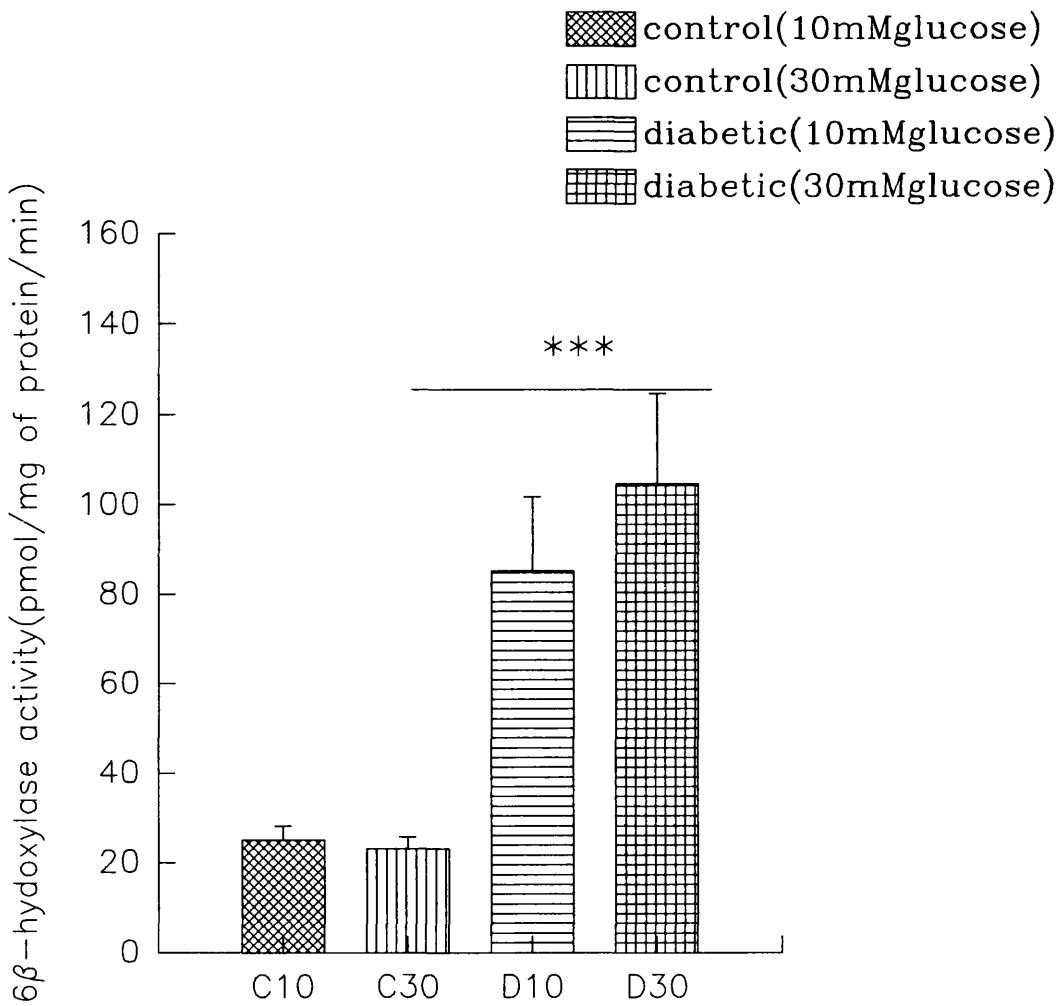


Figure 28. 6β-hydroxylase activity in control and streptozotocin- induced diabetic rat hepatocytes (in 10mM glucose and 30mM glucose). Values are the mean $\pm$ s.d for eight animals, p values relative to control values were obtained by ANOVA followed by Dunnet's test, *** $p < 0.001$.

105 ± 20 pmol/ mg of protein/ min ($p< 0.05$) compared to diabetic hepatocytes which were preincubated in 10mM glucose.

3. 8. 3. 16α- hydroxylase activity.

Figure 29 shows that a change in glucose concentration elicited no significant change in rat hepatocytes isolated from control or STZ-diabetic rats. In comparison with normal rat hepatocytes, enzyme activity in diabetic rat hepatocytes increased significantly ($p< 0.001$).

3. 8. 4. 17- oxosteroid oxidoreductase (17- OHSD) activity.

Unlike the effect of glucose on 7α-, 6β- and 16α-hydroxylase (cytochrome P450-dependent activities), the basal activity for 17-OHSD was reduced significantly ($p<0.001$) in normal rat hepatocytes pre incubated in 30mM glucose when compared to the activity in normal rat hepatocytes preincubation in 10mM glucose (basal 17-OHSD; 808 ± 30 pmol/ mg of protein/ min) (Figure 30). The activity was decreased significantly in diabetic hepatocytes ($p< 0.001$) compared to normal rat hepatocytes but no significant change was seen in 17-OHSD when diabetic hepatocytes were preincubated in 30mM glucose.

3. 8. 5. 5α- reductase activity.

An increase in this enzyme activity is seen with 30mM glucose preincubation (Figure 31) ($p< 0.05$) compared to the normal cells preincubated in 10mM glucose. There is no significant change in enzyme activity in diabetic rat hepatocytes compared to normal rat hepatocytes. The effect of glucose on diabetic hepatocytes as glucose concentration was increased to 30mM was not statistically significant.

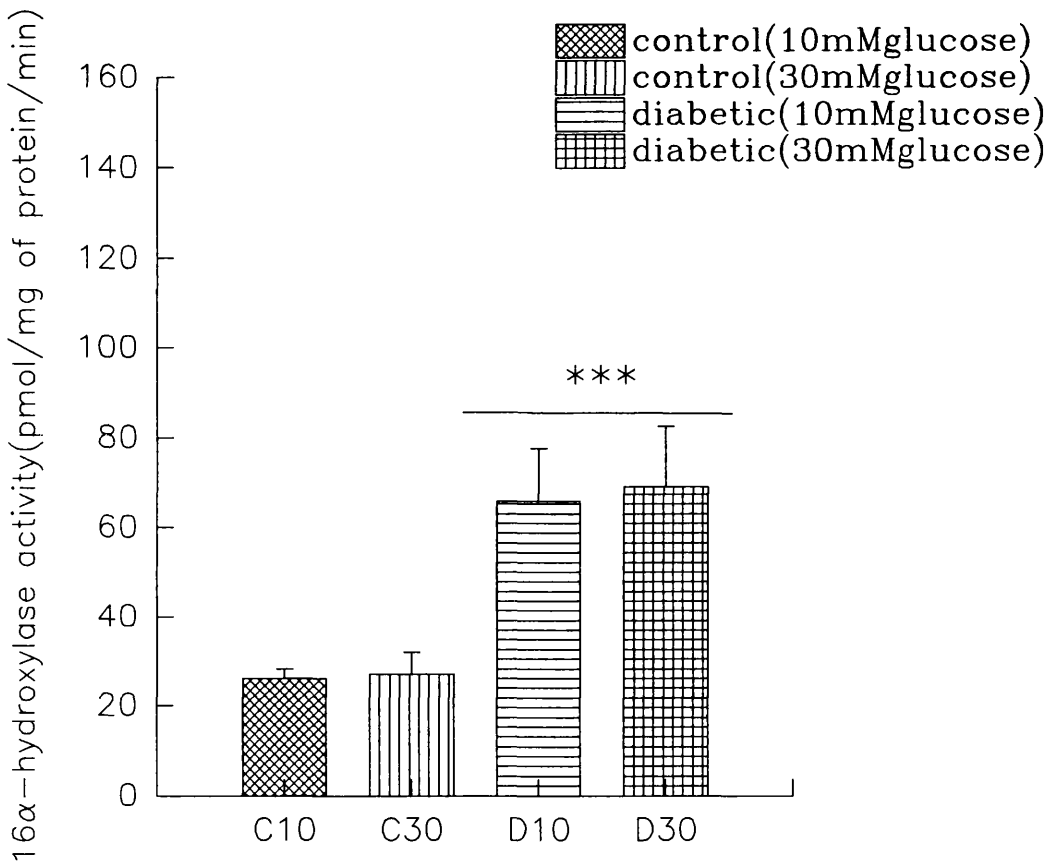


Figure 29. 16α-hydroxylase activity in control and streptozotocin- induced diabetic rat hepatocytes (in 10mM glucose and 30mM glucose). Values are the mean $\pm$ s.d for eight animals, p values relative to control values were obtained by ANOVA followed by Dunnet's test, *** $p< 0.001$.

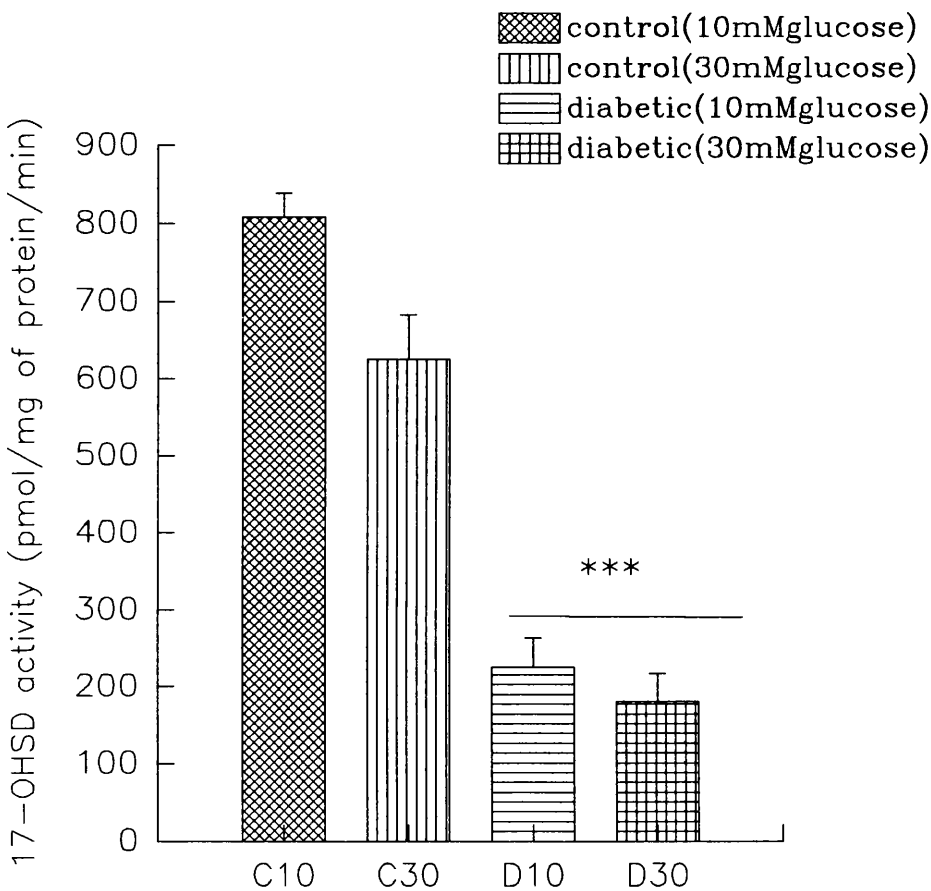


Figure 30. 17$\alpha(\beta)$- hydroxysteroid dehydrogenase activity in control and streptozotocin- induced diabetic rat hepatocytes (in 10mM glucose and 30mM glucose). Values are the mean $\pm$ s.d for eight animals, p values relative to control values were obtained by ANOVA followed by Dunnet's test, *** $p < 0.001$.

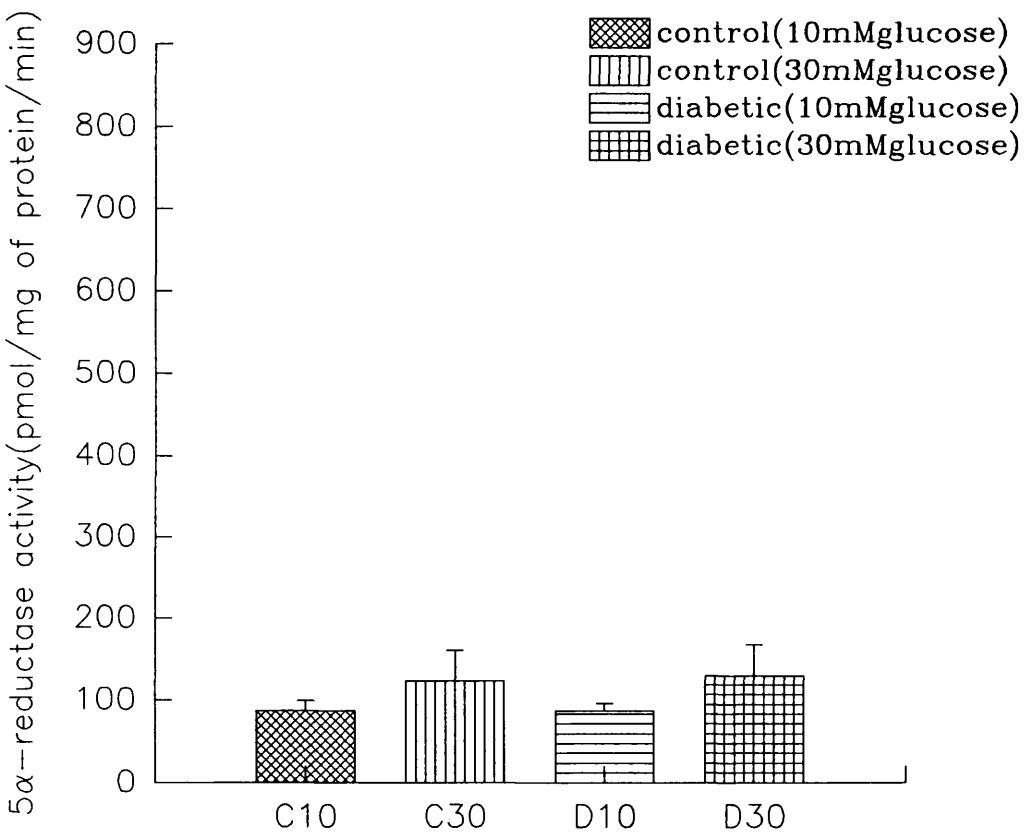


Figure 31. 5α- reductase activity in control and streptozotocin- induced diabetic rat hepatocytes (in 10mM glucose and 30mM glucose). Values are the mean $\pm$ s.d for eight animals, p values relative to control values were obtained by ANOVA followed by Dunnet's test.

3.9. THE EFFECT OF PHENETHYLBIGUANIDE, PHENETHYLGUANAMINE AND INSULIN ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN NORMAL RAT HEPATOCYTES.

Experiments were carried out to test the effect of phenethylbiguanide and phenethylguanamine as insulin mimetics on the activity of all enzymes metabolising androst- 4-ene- 3,17-dione in isolated male rat hepatocytes.

3. 9. 1. The effect of phenethylbiguanide and phenethylguanamine on 7α-hydroxylase activity

Phenethylbiguanide caused an increase in 7α- hydroxylase activity which exhibited an inverse U- shaped dose response curve with the maximum response seen at low concentration (10^{-9}M) (Figure 32). At the concentration 10^{-8}M to 10^{-6}M the activity of the enzymes diminished though still significantly higher than their respective control. There was no significant change in the activity when the biguanide was used at the lowest concentration (10^{-10}M) and highest concentration (10^{-5}M).

The cyclization of phenethylbiguanide to phenethylguanamine increased the effect of the drug on the 7α-hydroxylase activity significantly in a dose-dependent way. Maximum effect again was observed at low concentration (10^{-8}M) where activity was 712% of control. Above this concentration, the activity started to decline but phenethylguanamine still increased the activity of the enzyme significantly in all concentrations ($p< 0.01$) and increased the activity significantly more at concentrations of 10^{-8}M and 10^{-7}M ($p<0.001$) than phenethylbiguanide and insulin.

An increase in the 7α-hydroxylase enzyme activity was seen at insulin concentration from 10^{-10}M to 10^{-5}M. Maximum response of 665% of control was observed at the physiological concentration of 10^{-9}M. The percentage maximal

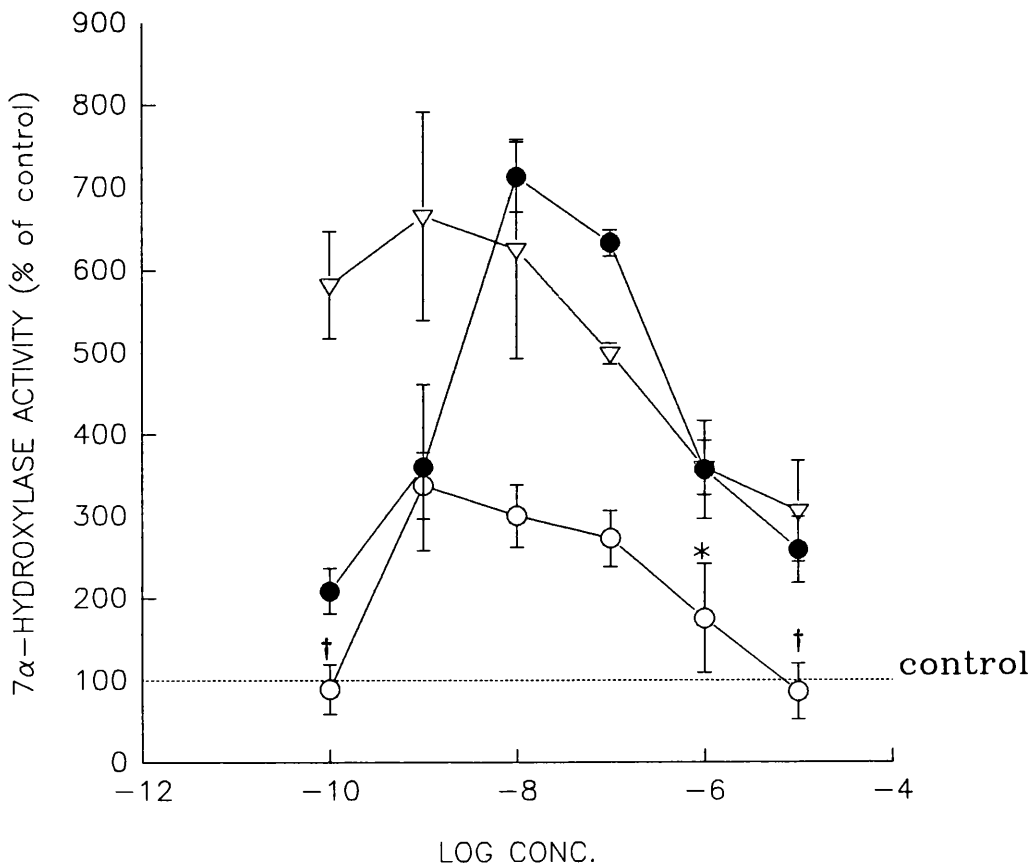


Figure 32. Effect of various concentrations of phenethylbiguanide (○), phenethylguanamine (●) and insulin (▽) on 7α- hydroxylase activity in normal rat hepatocytes compared to control (100%). The cells were preincubated with the compound and hormone for 30 min in Krebs- Henseleit medium. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal 7α- hydroxylase activity = 26 ± 7 pmol/ mg of protein/ min. All values $p<0.01$ except * $p<0.05$, † not significant.

response against control seemed to fall as the concentration of insulin increased above the physiological level (Figure 32) although the activity was still above control by 306% at the highest concentration (10^{-5}M).

3. 9. 2. The effect of phenethylbiguanide and phenethylguanamine on 6β hydroxylase activity.

Figure 33 shows the effect of phenethylbiguanide, phenethylguanamine and insulin on 6β- hydroxylase enzyme activity. The effect of phenethylbiguanide (10^{-10}M to 10^{-5}M) on the enzyme activity was found to display a bell- shape dose-response curve with the maximum effect observed at a concentration of 10^{-7}M and activity at the reported therapeutic concentration, 10^{-5}M, is not statistically significant ($p>0.05$). Phenethylbiguanide increased the activity significantly at the concentrations from 10^{-9}M to 10^{-7}M ($p< 0.01$) and at the concentration 10^{-6}M ($p< 0.05$) compared to control.

The effect was more pronounced with phenethylguanamine at all concentrations. At low phenethylguanamine concentrations (from 10^{-10}M to 10^{-8}M), the enzyme activity increased in a dose - dependent manner to 750% of control but at higher concentrations (10^{-7}M-10^{-5}M), there was less effect. Phenethylguanamine increased the activity significantly more at concentrations from 10^{-9}M to 10^{-6}M ($p< 0.001$) than phenethylbiguanide. When the same cells were preincubated with insulin, the activity of the 6β-hydroxylase significantly increased. It is interesting to note that insulin at its physiological concentration (10^{-9}M) gave the maximum response increase in the 7α- and 6β-hydroxylase activity. At 10^{-9}M insulin the activity increased significantly more compared to the effect of phenethylbiguanide and phenethylguanamine ($p< 0.001$).

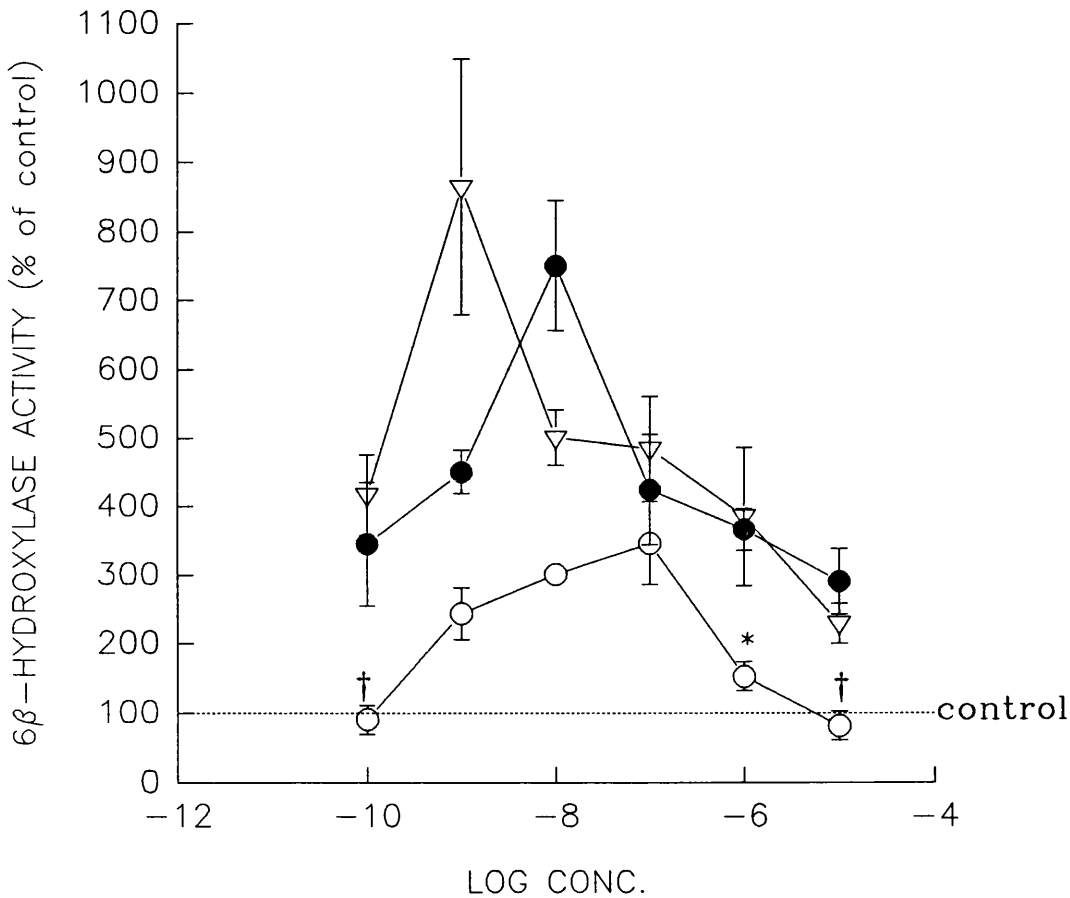


Figure 33. The effect of phenethylbiguanide (O), phenethylguanamine (●) and insulin (∇) on 6β- hydroxylase activity in normal rat hepatocytes compared to control (100%). The cells were preincubated with the compounds or hormone for 30 min in Krebs- Henseleit medium. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal 6β- hydroxylase activity = 22 ± 8 pmol/ mg of protein/ min. All values $p<0.01$ except $*p<0.05$, † not significant.

3. 9. 3. The effect of phenethylbiguanide and phenethylguanamine on 16α-hydroxylase.

16α- hydroxylase is one of the five enzymes which metabolises androst-4-ene-3,17- dione. It was found to increase in activity when incubated with phenethylbiguanide, phenethylguanamine and insulin. Phenethylbiguanide at lower concentrations(10^{-9}M to 10^{-7}M) caused a dose -dependent increase in the enzyme activity with maximum response at 10^{-7}M (Figure 34). At higher concentrations the effect of phenethylbiguanide was markedly reduced with activity returning to control levels at 10^{-6}M and 10^{-5}M while at the lower concentration of 10^{-10}M the activity was reduced significantly below control.

Phenethylguanamine still exhibited a dose- dependent effect on the enzyme activity at the lower concentrations of 10^{-10}M to 10^{-8}M. Maximum response was observed at 10^{-8}M which was 730% of control ($p< 0.01$; basal hydroxylase 16α activity 32 $\pm$ 5 pmol/ mg of protein/ min) with a subsequent fall in enzyme activity in the concentration range 10^{-7}M to 10^{-5}M. However activity was still significantly higher than respective controls. Phenethylguanamine increased the activity significantly more at concentrations of 10^{-9}M-10^{-7}M ($p<0.01$) than phenethylbiguanide. A significant increase in activity could be seen at insulin concentrations as low as 10^{-10}M but maximum response was shifted to the higher concentration of 10^{-7}M. However, at the highest concentration used 10^{-5}M enzyme activity decreased below the maximum but was still significantly above the control level.

3. 9. 4. The effect of phenethylbiguanide and phenethylguanamine on 17-hydroxysteroid dehydrogenase.

The effect of phenethylbiguanide, phenethylguanamine and insulin upon the activity of 17-hydroxysteroid dehydrogenase is shown in Figure 35.

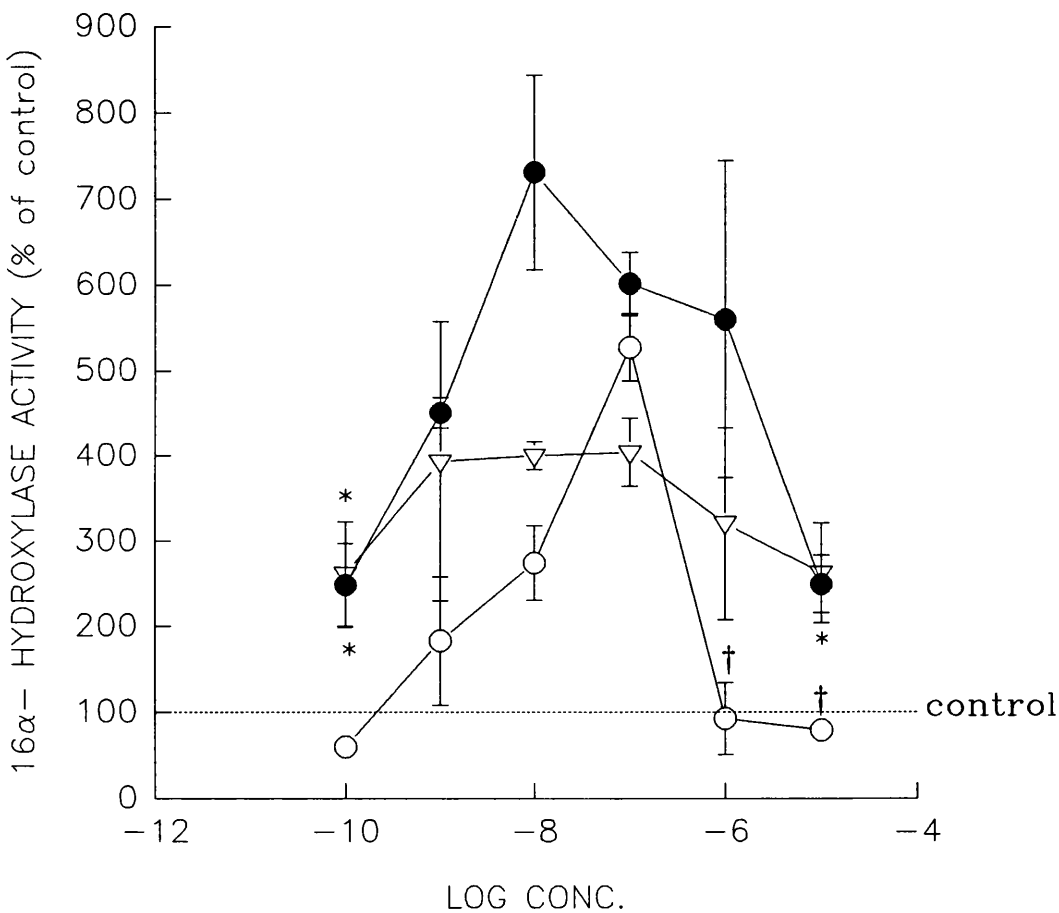


Figure 34. Effect of different concentrations of phenethylbiguanide (O), phenethylguanamine (●) and insulin (∇) on 16α- hydroxylase activity in normal rat hepatocytes compared to control (100%). The cells were preincubated with the compounds or hormone for 30 min in Krebs- Henseleit medium. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal 16α-hydroxylase activity = 32 ± 5 pmol/ mg of protein/ min. All values $p<0.01$ except * $p< 0.05$, † = not significant.

Phenethylbiguanide at the lower concentration of 10^{-10}M decreased the activity significantly ($p< 0.01$) to 38% of control. By increasing the concentration of drug to 10^{-9}M the activity of the enzyme increased significantly ($p<0.01$) to 251% of control. It appears that as the concentration of phenethylbiguanide increased (10^{-8}M to 10^{-5}M), there was a gradual decline in 17-hydroxysteroid dehydrogenase activity but the activity was still higher than the control.

In comparison, phenethylguanamine caused a dose-dependent decrease in activity at concentrations from 10^{-8}M to 10^{-5}M ($p<0.01$) (Figure 35). The enzyme exhibited a biphasic response towards insulin. A significant increase in enzyme activity was found at 10^{-10}M, whilst maximum increase of enzyme activity occurred at 10^{-9}M with a subsequent fall in enzyme activity at concentrations of 10^{-8}M and 10^{-7}M. However activity was still higher than with the control. At higher concentrations (10^{-6}M and 10^{-5}M), the activity decreased significantly below the control ($p< 0.01$).
It can be observed from Figure 35 that the effect of phenethylbiguanide, phenethylguanamine and insulin on the 17-hydroxsteroid dehydrogenase was not as pronounced as the effect on the 7-α, 6β- and 16α-hydroxylases.

3. 9. 5. The effect of phenethylbiguanide and phenethylguanamine on 5α-reductase activity.

A similar observation was noted for the 5α-reductase as seen for the 17-OHSD in that phenethylbiguanide increased the activity significantly ($p<0.01$) at concentrations of 10^{-8}M and 10^{-9}M. There was no significant change in enzyme activity observed at the lowest concentration of 10^{-10}M and at the highest concentration of 10^{-5}M, 10^{-6}M (Figure 36).

Phenethylguanamine increased the enzyme activity significantly at concentrations of 10^{-10}M, 10^{-9}M, 10^{-7}M and 10^{-6}M ($p< 0.05$). There was no significant change in enzyme activity at concentrations of 10^{-8}M and 10^{-5}M. Insulin at the lower concentrations of 10^{-10}M and 10^{-9}M elicited increases in enzyme activity

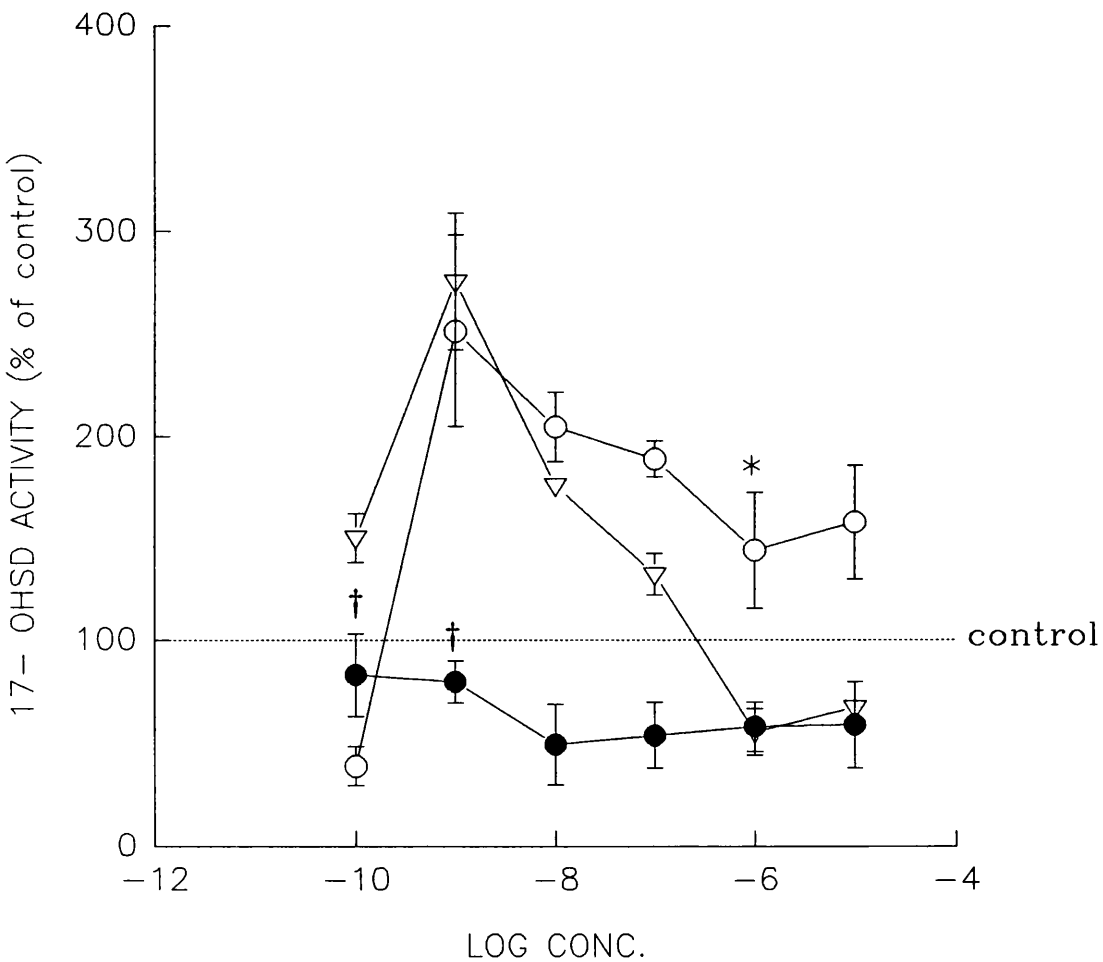


Figure 35. Effect of different concentrations of phenethylbiguanide (O), phenethylguanamine (●) and insulin (∇) on 17α(β)- hydroxysteroid dehydrogenase activity in normal rat hepatocytes compared to control (100%). The cells were preincubated with the compounde and hormone for 30 min in Krebs- Henseleit medium. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal 17α(β)- hydroxysteroid dehydrogenase activity = 346 ± 32 pmol/ mg of protein/ min. All values $p<0.01$ except * $p< 0.05$, † = not significant.

with the maximum effect again seen at 10^{-9}M (the activity rose to 703% of the control value). At the concentrations of 10^{-8}M and 10^{-7}M , the effect of insulin was markedly reduced despite still being significantly greater than the respective control ($p<0.01$) with activities of 400% and 341% of control respectively. High concentrations of insulin ($>10^{-5}$M) gave no significant effect.

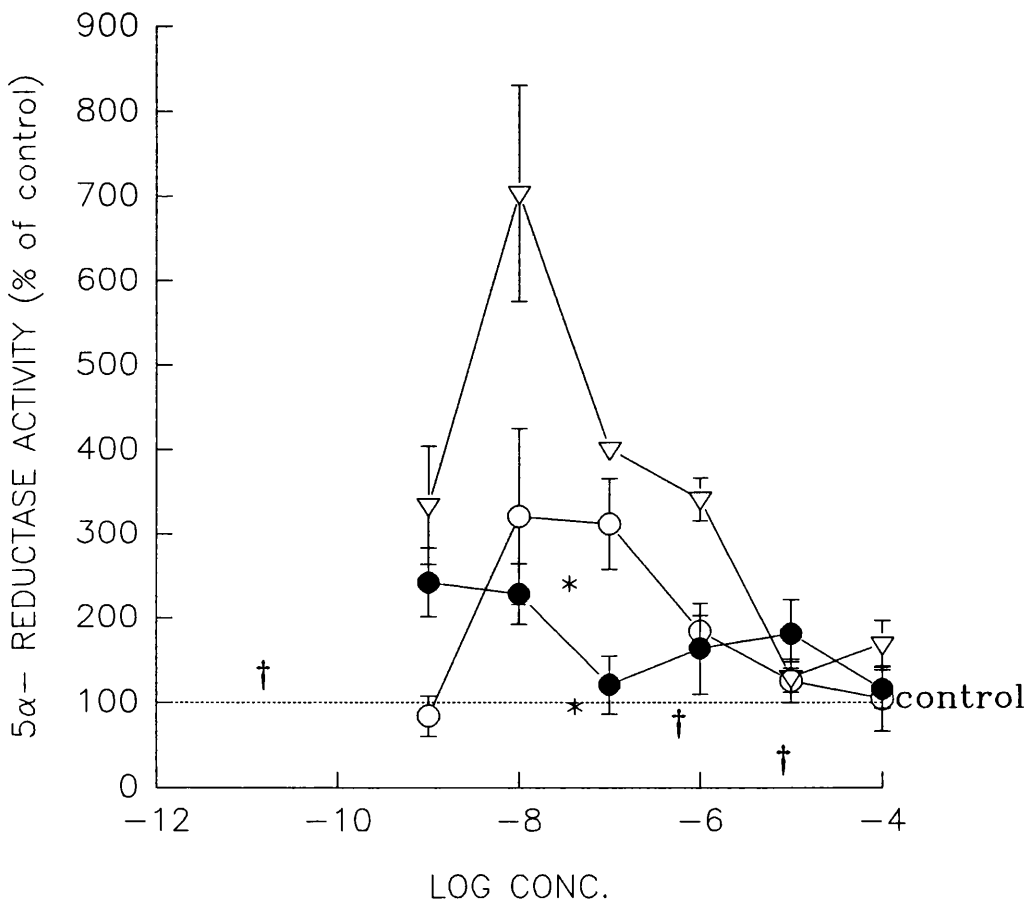


Figure 36. Effect of various concentrations of phenethylbiguanide (O), phenethylguanamine (●) and insulin (∇) on 5α- reductase activity in normal rat hepatocytes compared to control (100%). The cells were preincubated with the compounds or hormone for 30 min in Krebs- Henseleit medium. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnett's test. Basal 5α- reductase activity = 92 ± 22 pmol/ mg of protein/ min. All values $p<0.01$ except * $p< 0.05$, † = not significant.

DISCUSSION

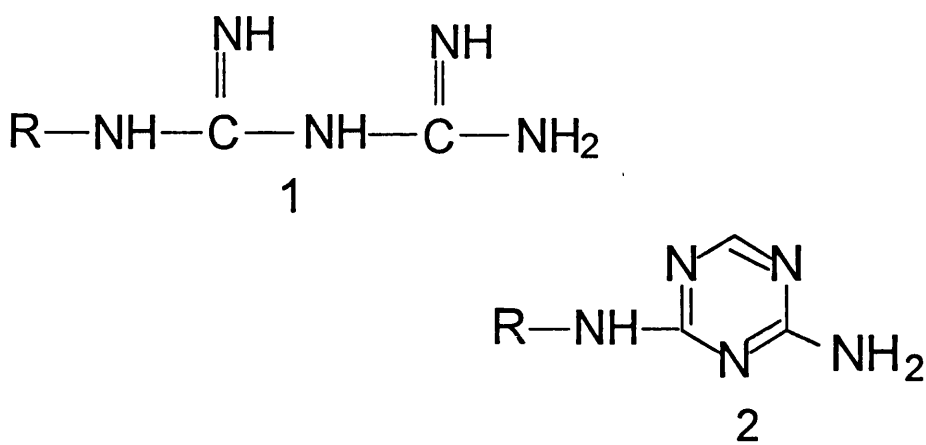
4. 1. The effect of biguanides guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in normal rat hepatocytes

Hepatic glycogen metabolism provides a significant contribution towards glucose homeostasis. Glycogen breakdown in the liver releases glucose into the blood, thus, raising serum glucose levels. Conversely, excess glucose can be taken into the liver and converted to glycogen. The synthesis and breakdown of glycogen are controlled essentially by the two enzymes, glycogen synthase and glycogen phosphorylase respectively. Glycogen phosphorylase, which catalyzes the first step in the intracellular degradation of glycogen (see Introduction 1.3.2), is the rate limiting enzyme for glycogenolysis (Cao *et al.*, 1993). The enzyme can exist in two different forms in the liver the b form, which is phosphorylated and essentially inactive, and the a form, which is not phosphorylated and is active. Conversion from one form to the other is controlled by a cascade mechanism involving cAMP and a series of kinases (Figure 2). Glycogen phosphorylase has been shown to decrease in diabetic rat hepatocytes and the enzyme activity was restored to control values by insulin therapy (Khandelwal *et al.*, 1977; Studer & Ganas, 1989). Many studies have shown that glycogen metabolism in liver is regulated by insulin, which gives a suppression of hepatic glycogen phosphorylase in *in vivo* and *in vitro* studies (Hers *et al.*, 1973; VandeWerve *et al.*, 1977; Hartmann *et al.*, 1987; Ruiz *et al.*, 1993).

It was observed that insulin inhibited phosphorylase activity in liver by two separate mechanisms. In liver cells containing normal levels of calcium, it inhibits α-adrenergic activation of phosphorylase by decreasing the net mobilisation of Ca^{2+} ions from intracellular stores thus diminishing the cytosolic Ca^{2+} and hence stimulation of phosphorylase kinase. In calcium- depleted hepatocyte , however, it antagonises α-adrenergic action by inhibiting the accumulation of cAMP (Blackmore *et al.*, 1979). Insulin, at physiological concentrations, decreases the phosphorylation state of the phosphorylase and phosphorylase kinase in skeletal muscle (Zhang *et al.*, 1989) and it

is thought that may also happen in liver. Thus the activity of glycogen phosphorylase is important for the maintenance of serum glucose concentrations and can be controlled by insulin as part of its regulation of glucose homeostasis.

The biguanides, 1,1-dimethylbiguanide (metformin) and phenethylbiguanide (phenformin) have been used for several decades to lower the elevated glucose levels in patients with non-insulin-dependent (type II) diabetes mellitus (Hermann, 1979; Schafer, 1983; Bailey & Nattrass, 1988; Bailey, 1992). They are said to act mainly by decreasing hepatic glucose production but the cellular mechanisms responsible for these effects have not been resolved (Bailey, 1993). The cyclization of biguanide to the corresponding 1,3,5-triazine (guanamine) has previously been shown to give compounds which still lower blood glucose levels in normal as well as diabetic rats (Brzozwski *et al.*, 1980 a, b; Gajewski *et al.*, 1983 a, b; Gajewski *et al.*, 1984; Tamasdan et al., 1987). In this series of experiments, a novel aspect of the beneficial effect of biguanides and guanamines in diabetes was explored by investigation of the effect of the compounds on the activity of glycogen phosphorylase a in normal and diabetic rat hepatocytes as a measure of insulin-like activity.



IX

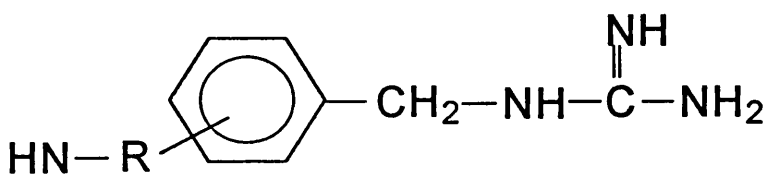
Phenethylbiguanide (IX, 1, R= C6H5[CH2]2) significantly inhibited the activity of glycogen phosphorylase a with the maximum effect observed at 10^{-4}M. Phenethylbiguanide at a concentration of 4.8 x 10^{-4}M has previously been shown to

reduce glucose output by half, despite increasing glycogenolysis in adult liver culture (Brown *et al.*, 1975). After oral treatment, the concentrations of phenethylbiguanide in the plasma of human diabetics are in the range of 1-2 x 10^{-6}M (Hermann & Melander, 1992; Bailey, 1993). In the present studies the effect of phenethylbiguanide could be seen at 10^{-6}M. These observations raise the possibility that phenethylbiguanide could exert part of its blood glucose lowering effect via a direct effect on glycogen phosphorylase a in liver.

Phenethylbiguanide decreases the intracellular cyclic AMP content of isolated hepatocytes (Luly *et al.*, 1977) analogous to the action of insulin. It can be inferred therefore that the inactivation of glycogen phosphorylase could be related to the decrease of intracellular cyclic AMP concentration. Recently Ubl - Joachim and co - workers (1994) demonstrated an effect of biguanides on phenylephrine -induced $[Ca^{2+}]i$ oscillations in rat hepatocytes by a direct negative interference with the IP_3(inositol-1,4,5-trisphosphate)-sensitive Ca^{2+}-channel. IP_3-induced release of Ca^{2+} from intracellular stores could be diminished by prior addition of biguanides. It may be that biguanides influence the agonist - induced $[Ca^{2+}]i$ oscillations predominantly by altering the cyclic cAMP signalling pathway. It has been shown that activation of cAMP-dependent protein kinase via increasing concentrations of cAMP potentiates IP3-induced Ca^{2+} mobilisation in permeabilized rat hepatocytes (Burgess *et al.*, 1991; Hajnoczky *et al.*, 1993) and increases the frequency of phenylephrine induced $[Ca^{2+}]i$ oscillation (Somogyi *et al.*, 1992; Sanchez-Bueno *et al.*, 1993). From our finding on the effect of phenethybiguanide on glycogen phosphorylase a we can speculate that the effect of phenethylbiguanide on the phosphorylase enzyme may be through one of these mechanisms.

A clear structure-activity relationship was observed with the synthesised biguanides, the most active compound of the biguanide series was benzylbiguanide (structure IX, 1, R= $C_6H_5CH_2$), this compound was equal to insulin in activity, in agreement with that observed *in vivo* (Paul *et al.*, 1963). When R is benzyl the hypoglycaemic activity of the compound is found to be greatly increased from that of

the phenethyl derivative. Furthermore benzylguanidines carrying an amino group have been shown to have hypoglycaemic activity in mice with intact metabolism (Pfeiffer & Sarrazin, 1985) (structure X) indicating the effectiveness of the guanidino group in lowering blood glucose levels..

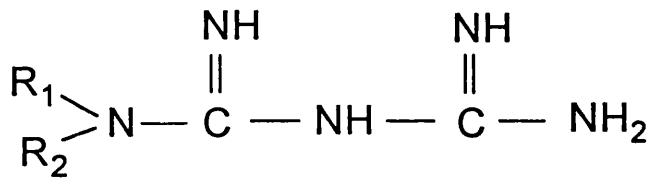


X

Moderate activity was shown by the compound having a phenyl group substitution (structure IX, 1, R= C6H5). The least active compound of this series was the phenethyl group substitution in R_1(I) (i.e. the clinically used oral hypoglycaemic, phenformin, is the least active).

Cyclization of the compounds to the corresponding 1,3,5-triazines (structure IX,2) still gives compounds which inhibit the activity of glycogen phosphorylase. Our results confirm the idea that stabilisation of the biguanide group by cyclization to a triazine might result in enhanced antidiabetic potency. In the guanamine series of compounds, benzylguanamine (structure IX, 2, R= $C_6H_5CH_2$) had the highest potency (similar to insulin) as compared with substitution with the phenyl (structure IX, 2, R= C_6H_5) or phenethyl group (structure IX, 2, R= $C_6H_5[CH_2]_2$) .

The idea that cyclisation of the biguanide is required for activity can be tested by comparing the effects of phenformin and metformin. 1,1-dimethylbiguanide (metformin) (structure XI, 1, R_1,R_2 = CH_3) was more potent than phenethylbiguanide (phenformin) on the enzyme activity in normal hepatocytes. This finding was in agreement with other work which has demonstrated that alkylbiguanides are more active than aromatic biguanides in the action on carbohydrate metabolism in rats (Piccinini *et al.*, 1960).



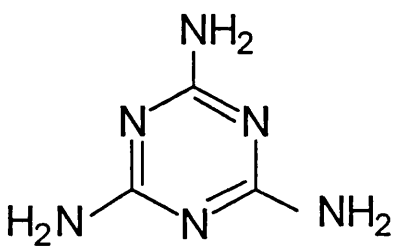
XI

1,1-dimethylbiguanide, however, cannot became cyclic and thus, cyclization may not be necessary for action. The results of this investigation are in agreement with results from another laboratory on the possible mechanism of 1,1-dimethylbiguanide action on glycogen metabolism (Jacobs *et al.*, 1987). Gullo *et al* (1988) have suggested that 1,1-dimethylbiguanide improves insulin mediated [^{14}C]-glucose incorporation into glycogen in cells isolated from the liver (Melin *et al.*, 1990). Taken together these results suggest that biguanides activate liver glycogen synthase and also inactivate phosphorylase in cells isolated from the liver which is an organ of considerable importance with respect to body glucose homeostasis as discussed above.

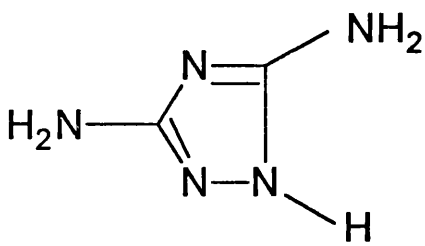
In this effect the biguanides (phenformin and metformin) seem to be unique relative to other hypoglycaemic agents as only insulin has been shown to have effects like biguanides on the activity state of glycogen phosphorylase a (Hartmann *et al.*, 1987). Sulfonylureas have, however, been found to have interesting effects in isolated cell systems (Cabello *et al.*, 1987; Lopez-Alarcon *et al.*, 1988; Lopez-Alarcon *et al.*, 1990) but it does not appear that the mechanism of action of biguanides is similar to that of sulfonylureas. Mojena *et. al.* (1989) have demonstrated that sulfonylureas exert a paradoxical activation of glycogen phosphorylase a, possibly by a calcium mediated mechanism. As a consequence of this stimulation of glycogenolysis, the cellular concentrations of hexose 6-phosphate and fructose-1,6- diphosphate are increased. They also suggested that sulfonylureas direct the glycogenolytic flux mainly to lactate, and to a lesser extent to glucose. Alarcon *et al.* (1993) confirmed the hypothesis that the activation of glycogen phosphorylase by sulfonylureas occurred as a result of an increase in hepatocyte [Ca^{2+}]i and the subsequent allosteric effect of this ion on glycogen phosphorylase kinase activity (Khoo & Steinberg, 1975).

In a further attempt to ascertain which part of the biguanide and/or guanamine structure may be involved in its potential hypoglycaemic (glycogen phosphorylase lowering) effect, a variety of other compounds were tested which had some structural similarity with biguanides and/or guanamines or had been shown previously to have effects on blood glucose levels.

The guanamine structure contains a six-membered ring containing three nitrogen atoms (a 1,3,5-triazine) and the biguanides are thought to be able to cyclize by hydrogen bonding *in vivo* to give what looks like a triazine but is, in effect, a five-membered ring resembling a 1,2,4-triazole. It was decided to try the effects of a 1,3,5-triazine and a 1,2,4-triazole on glycogen phosphorylase activity. Melamine (2,4,6-triamino-1,3,5-triazine) (structure XII) was compared with 3,5-diamino-1,2,4-triazole (structure XIII). Melamine completely inhibited enzyme activity but this was found to be due to its cytotoxic action. The triazole, however, exhibited glycogen phosphorylase lowering activity similar to the biguanides and guanamines From these results it may be concluded that the triazine ring with 2-amino groups may be all that is required for the hypoglycaemic activity. This points us towards an idea about the future work on cyclization of biguanides to triazole rings instead of triazines



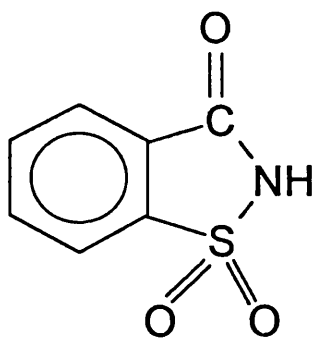
XII



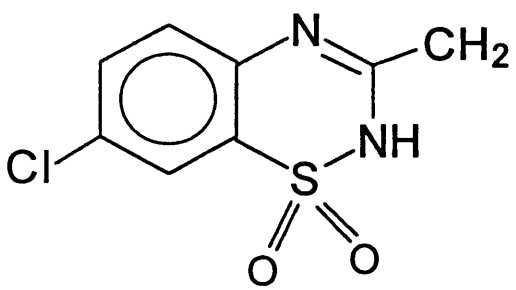
XIII

In earlier *in vivo* work, it had been shown that saccharin could lower blood glucose levels (Debbri, 1995) and that the structures of the "cyclised" phenformin and saccharin could be overlapped with convergence of their charged groups (using a molecular graphics package, CHEMMOD, developed in the Department of Chemistry, University of Glasgow). We, thus, decided to compare the effects of phenformin and

saccharin on glycogen phosphorylase a activity. This was done in spite of the earlier evidence that sulphonylureas have a paradoxical effect on glycogen phosphorylase (they increase its activity (Mojena et.al., 1989)) and saccharin more resembles the sulphonylureas, having a cyclic sulphonamide ring structure (structure XIV). In a comparison between phenethylbiguanide (phenformin) and o-sulfobenzimide (saccharin), which is also used as a sweetening agent, the latter was shown to possess glycogen phosphorylase lowering properties and behaves in this respect like phenformin, whilst having no obvious structural resemblance (see sructure IX and XIV). This suggests that the guanadino group is not strictly necessary for the activity seen in this study, since the cyclic sulfonamide group in saccharin also had the same effect on glycogen phosphorylase activity. However, the guanadino group did have more effect on the activity than the cyclic sulfonamide group. It may be that the charge distribution over the molecule is of greater importance than the actual functional group.



XIV



XV

Diazoxide, which contains a 6-membered cyclic sulphonamide ring (structure XV) was used to clarify the structure-activity relationship by comparing its effects to saccharin which contains a 5-membered ring with the same group. Diazoxide is an antihypertensive, with potent hyperglycaemic actions when given orally. Hyperglycaemia is reported to result primarily from inhibition of insulin and proinsulin

secretion (Levin *et al.*, 1975; Leahy, 1993). Diazoxide interacts with an ATP-sensitive K^+ channel and either prevents its closing or prolongs the open time. This effect is opposite to that of the sulfonylureas (Misler *et al.*, 1989; Panteu *et al.*, 1989; Moreau et al., 1992). The drug does not inhibit insulin synthesis, and thus there is an accumulation of insulin within the ß-cell. Diazoxide also has a modest capacity to inhibit peripheral glucose utilisation by muscle and to stimulate hepatic gluconeogenesis. This effect is opposite to that of the biguanides (Bailey & Puan, 1985). These result demonstrate that the oral hyperglycaemic agent diazoxide activated glycogen phosphorylase a in contrast to saccharin which inhibited its activity. These results suggested that it is not the presence of a cyclic sulphonamide group that decreases the activity of glycogen phosphorylase a but, perhaps, the resemblance to sulphonylureas that is lost in diazoxide.

CONCLUSIONS

1. All of the synthesised biguanides and guanamines mimic insulin in decreasing glycogen phosphorylase a activity.
2. In the biguanide series, the benzyl and phenyl derivatives were more potent than the phenethylbiguanide (phenformin).
3. Cyclization of the biguanides to the corresponding 1,3,5- triazines (guanamines) still give compounds which inhibit the activity of glycogen phosphorylase
4. In the cyclized guanamine series, the most potent was benzylguanamine, which had a potency similar to insulin.
5. Cyclization does not seem to be strictly necessary for action, however, as metformin which does not cyclise can also decrease glycogen phosphorylase activity.

6. The triazine ring with 2- amino groups is all that is required for the hypoglycaemic activity. This can be tested in future work on cyclization of biguanides to triazol rings instead of triazines.
7. It is not only biguanides and guanamines that can have this effect, saccharin (a cyclised sulphonamide 5 membered ring) also decreases glycogen phosphorylase whereas diazoxide (a cyclised sulphonamide 6 membered ring) increases the same activity.

4. 2. Effects of biguanides, guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in cultured hepatocytes from STZ diabetic rats.

Since biguanides and guanamines have been shown to have an influence on the glycogen phosphorylase a activity (previous section) as insulin mimetics in normal rat hepatocytes, it is pertinent for us to extend our investigation to the effect of biguanides and guanamines on hepatocytes isolated from the diabetic rat. In order to discuss the possible mechanism of action of biguanides, guanamines and insulin in altering the activity of phosphorylase a, it is necessary first to look at the nature of the enzyme itself and how the diabetic state may affect the enzyme activity.

Glycogen phosphorylase a, is a key enzyme in the regulation of glycogen metabolism as discussed previously and its activity has been shown to decrease in diabetic rats (Khandelwal *et al.*, 1977). A decrease in activity in diabetic rats was also observed by Bahnak & Gold (1982) when assayed by the method of Stalmans and Hers (1975). Similarly, total phosphorylase shows a significant decrease in activity in rats after injection with alloxan to induce diabetes when assayed by the same method as above (Miller, 1978) and by the high substrate (Glc-1-p) assay using liver homogenates from streptozotocin diabetic rats (Tan & Nattal, 1975).

The activities of glycogenolytic enzymes, i.e. phosphorylase (both a and b), phosphorylase kinase and protein kinase (in the presence or in the absence of cyclic AMP), were significantly decreased in diabetic animals. The enzyme activities were restored to control values by insulin therapy. Glycogen synthase (I- form) activity, similarly decreased in diabetic animals and was also restored to control values after the administration of insulin (Khandelwal *et al.*, 1977). The same results were found in the STZ diabetic hepatocytes in the acute model in that there was a decrease in glycogen phosphorylase activity and a corresponding reduction in cytosolic free calcium. corrected by insulin replacement (Studer & Ganas, 1989).

In STZ diabetic liver, phosphorylase (a and total) activities were significantly lower than those seen in normal animals during fasting and re-feeding. cAMP-dependent protein kinase and phosphorylase kinase activities also showed substantial reduction during fasting and subsequent feeding (Pugazhenthi & Khandelwal, 1991). In primary cultured hepatocytes, diabetes results in a loss of total phosphorylase activity similar to, although more pronounced than, that found in isolated perfused liver (Miller *et al.*, 1986). Rulfs *et al.* (1989) demonstrated that the rate of glycogen phosphorylase synthesis is significantly decreased in diabetic hepatocytes compared with normal rat hepatocytes. No significant difference was seen in the rate of phosphorylase degradation in these cells. The decrease in the synthetic rate corresponds well with observed decreases in phosphorylase activity measured in these cells (Bahnak & Gold, 1982) as well as in other liver preparations (Miller, 1978; Roesler & Khandelwal, 1987). Reports from Roesler and Khandelwal (1986 a) on the diurnal rhythm of liver phosphorylase a activity in normal and genetically diabetic mice have indicated that total phosphorylase (a and b forms) is mainly governed by a change in enzyme protein concentration. Results from the same laboratory also confirm this relationship in rat liver (Roesler & Khandelwal, 1987).

In this study, the activity of glycogen phosphorylase a was lower in diabetic animals than in control or insulin-treated diabetic animals but there was no difference between the latter two groups. This result is in agreement with the observations of

Khandelwal *et al.*, 1977. In the liver of diabetic rats, a decrease in total phosphorylase, phosphorylase a (Langdon & Curnow, 1983) and hormonal activation of the enzyme (Rodriguez *et al.*, 1989) should spare liver glycogen stores. However, such a sparing effect does not occur since liver glycogen and phosphorylase a decrease together as shown by Lavoie and VendeWerve (1991). The explanation for this paradox is probably that the lowered synthase phosphatase activity found in diabetes leads to an impairment in the activation of glycogen synthase and consequently to a reduction in glycogen synthesis despite low phosphorylase activity (Stalmans *et al.*, 1987). The lower basal phosphorylase a could be the result of increased phosphorylase phosphatase activity due to low concentration of its inhibitor, glycogen (Stalmans *et al.*, 1971) and this explains why basal phosphorylase a is lower in diabetic hepatocytes.

The results of this study on diabetic hepatocytes indicate that biguanides and guanamines have insulin - like effects on hepatic phosphorylase a i.e. an increase in enzyme activity. The effect is opposite to that seen in hepatocytes from control animals discussed above and may be compensation for the inhibition of glycogen phosphorylase activity seen in liver from diabetic animals (Khandelwal *et al.*, 1977; Pugazhenthi & Khandelwal, 1991) which is perhaps due to the low amount of glycogen seen in liver of these animals as discussed above (Lavoil & VendeWerve, 1991).

The activation of glycogen phosphorylase a elicited by phenethylbiguanide was in a non dose-dependent manner, approximately maximal effect was obtained when drug was used at 10^{-6}M. Phenethylbiguanide at this concentration acts directly to fully mimic the proteolytic inhibitory effect of insulin in the absence of extracellular hormone. The proteolytic process inhibited by insulin was identified as the lysosomal pathway in Langendorff-perfused rat hearts (Thorne & Lockwood, 1990). The concentrations of phenethylbiguanide employed to lower blood glucose in man are approximately $3x10^{-6}$M (Schafer, 1983) - similar to the concentrations of drug giving maximal effects in this study.

The presence of the phenyl and benzyl group as a substitution does not increase the enzyme activity in diabetic hepatocytes as much as a phenethyl group. In contrast to normal rat hepatocytes, diabetic hepatocytes showed the greatest response to phenethylbiguanide and only moderate effects with benzylbiguanide. This confirms the previous observations in non - diabetic animals and man (Herman, 1979; Billingham *et al.*, 1981; Bailey & Broabent, 1981; Lord *et al.*, 1983).

Cyclization of the biguanides to the corresponding 1,3,5- triazine gave only small changes in activity. In the guanamine series of compounds, phenethylguanamine had the highest potency (more effect than insulin) as compared with substitution with the phenyl or benzyl group. This observation contrasts with the observation in the normal rat hepatocytes where benzylguanamine was more effective on the glycogen phosphorylase than phenethylguanamine. In the cyclization of phenethylbiguanide to phenethylguanamine, there was a tendency for diabetic hepatocytes to become more responsive to phenethylguanamine than phenethylbiguanide at low concentrations. We can concluded that the biguanides and guanamines still mimic insulin in their effect on glycogen phosphorylase. However, in contrast to normal cells, the structure-activity relationship was reversed.

Benzylbiguanide > phenylbiguanide > phenethylbiguanide (normal)
Phenethylbiguanide > phenylbiguanide > benzylbiguanide (diabetic)
Benzylguanamine > phenylguanamine > phenethylguanamine (normal)
Phenethylguanamine > phenylguanamine > benzylguanamine (diabetic)

Both phenethylbiguanide (phenformin) and 1,1-dimethybiguanide (metformin) stimulate the activity of glycogen phosphorylase a under similar conditions but the cells from diabetic rats were much more responsive to phenethylbiguanide. This effect is opposite to that which was observed in normal rat hepatocytes where 1,1-dimethylbiguanide was more effective. This relationship indicates that the cyclization of biguanides may be necessary for action in diabetic hepatocytes and also that

phenethylbiguanide may exert its anti-diabetic actions through cyclization in body fluids. This result on the effect of biguanides on glycogen phosphorylase in diabetic hepatocytes was in agreement with what Reddi and Jyothirmayi (1992) who observed that hepatic and skeletal muscle glycogen phosphorylase activity is significantly lower in diabetic than in control. This decrease has also been reported in alloxan and streptozotocin diabetic rats (Khandelwal, *et al.*, 1977; Pugazhenthi & Khandelwal, 1990) where diabetic mice were treated orally with biguanides for 28 weeks, which stimulated the activity of glycogen phosphorylase a in the liver, whereas it did not have any effect on the muscle enzyme. Since phosphorylase a is involved in the breakdown of glycogen, biguanides enhance glycogen breakdown in the liver of diabetic rats. Stimulation of both glycogen synthase and phosphorylase a by biguanides may thus cause increased turnover of hepatic glycogen.

Further structure-activity relationship studies on diabetic hepatocytes by the use of s-triazine demonstrated that the presence of a triazine ring in the guanamine structure may be important for the activity since the presence of a triazine ring increased the activity of glycogen phosphorylase in diabetic rat hepatocytes to 137% of control. A similar effect was observed when 3,5-diamino-1,2,4-triazole was used. This result suggests that the triazine ring part of the guanamine may be all that is required for activity and suggests that future work on cyclization of biguanides to triazole rings instead of triazine rings is still required to complete the investigation.

The effect of o-sulfobenzimide (saccharin) on diabetic hepatocytes was also different to that seen in hepatocytes from normal animals. In diabetic hepatocytes, saccharin had no significant effect on the cells. From this result we can deduce clear differences in the effect on the glycogen phosphorylase activity between the guanadino group in phenethylbiguanide and the cyclic sulphonamide group in saccharin unlike the similar effects seen in normal cells, since the 5-membered cyclic sulphonamide does not affect the activity and seems to have no significance.

It was also clearly demonstrated that, as with insulin, there is no improvement in the activity of the enzyme with either saccharin, which contains a 5 membered cyclic

sulphonamide, or diazoxide, which contains a 6 membered ring cyclic sulphonamide. This result confirms that both compounds (in diabetic hepatocytes) exert little effect and the presence of a 5 or 6 membered cylic sulphonamide does not improve the activity. In fact it appears that, in the diabetic hepatocytes the guanidino group may possess the most significant hypoglycaemic activity.

CONCLUSION

1. The effect of synthesised biguanides and guanamines mimics insulin in that they increase glycogen phosphorylase a activity in diabetic hepatocytes as opposed to the effect seen in hepatocytes from control animals.

2. In the biguanide series, the phenethylbiguanide was more potent than the phenyl and benzyl derivatives in contrast to the effect in normal rat hepatocytes.

3. Cyclization of the biguanide to the corresponding 1,3,5- triazine (guanamines) still gave compounds which increase the activity of glycogen phosphorylase.

4. In the cyclized guanamine series , the most potent was phenethylguanamine, which had a potency more than insulin in contrast to the effect seen in normal rat hepatocytes.

5. Cyclization may be necessary for action since phenformin showed more effect than metformin , again in contrast to the effect in normal rat hepatocytes.

6. The triazine ring seems to be all that is required for the hypoglycaemic activity but cyclization of biguanides to a triazole ring may still be worth investigating.

7. The guanadino group is necessary for the activity on diabetic hepatocytes since the cyclic sulphonamides (saccharin and diazoxide) do not possess any significant effect on diabetic cells.

4. 3. Effect of biguanides guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in isolated hepatocytes from insulin treated diabetic rat.

In the previous series of experiments it had been shown that the biguanides and guanamines had insulin-like effects on the activation of glycogen phosphorylase a in diabetic hepatocytes, which may be a compensation for the inhibition of glycogen phosphorylase seen in the liver from diabetic rats. Insulin treatment of STZ- diabetic rats is known to reverse other diabetes-related parameters, such as the increase in serum glucose levels, back to control levels. In this study we have tried to extent our experiments to see the effect of biguanides and guanamines on the activity of glycogen phosphorylase in cells isolated from insulin treated diabetic rats to see if the effects seen in control animals could be restored. It was previously observed that the defect in the activity of glycogen phosphorylase in the liver of alloxan- and streptozotocin-diabetic rats can be repaired by injection of insulin *in vivo* (Miller *et al.*, 1986). This supports the contention that the defect is due to insulin deficiency rather than a hepatotoxic effect of alloxan or streptozotocin (Miller, 1978; Haverstick et al, 1979; Bollen & Stalmans, 1984).

Our results, comparing the activity of glycogen phosphorylase a in streptozotocin-induced diabetes, insulin treated diabetic and control, showed that there is no significant difference between the control and the insulin-treated diabetic group. Khandelwal and co-workers (1977) also demonstrated that there is no significant difference in protein kinase and phosphorylase kinase activities between the control and insulin-treated diabetic rat.

This study has also clearly demonstrated that phenethylbiguanide actually inactivates glycogen phosphorylase in hepatocytes isolated from insulin-treated diabetic rats in a dose-dependent manner. This effect is in contrast to the effect of the compound on normal rat hepatocytes where there is little enhancement in the activity with increasing concentrations and in hepatocytes isolated from diabetic rats where a

significant increase in enzyme activity was seen. The effect of phenformin is, thus, similar but not identical to that seen in the control animals.

Examination of the effect of the side-chain by changing the phenethyl to the phenyl or benzyl group indicates that phenyl and benzyl substitution leads to an increase in the activity of the compound. The same effect was observed when the activity relationships in the normal hepatocytes was studied. In comparing the effects of the biguanides on hepatocytes isolated from insulin-treated diabetic rats, it was found that the most potent compound in this series was benzylbiguanide as in normal rat hepatocytes. With the cyclization of biguanides to guanamines there is no clear improvement in the activity of the compounds on the same cells under similar experimental conditions. This is again similar, though not identical, to the changes in activity observed in the cells from the normal animals.

The result of a comparison between phenethylbiguanide (phenformin) and 1,1-dimethylbiguanide (metformin) demonstrated that the cells from insulin treated diabetic hepatocytes were more sensitive to the 1,1- dimethylbiguanide than to the phenethylbiguanide (phenformin). This effect was similar to that in normal rat hepatocytes but it appears clearer in the insulin-treated diabetic hepatocytes. From this result we can concluded again that cyclization may not be entirely necessary for action in this type of cells.

The triazole ring in 3,5-diamino-1,2,4-triazole possesses the same effect as guanamines, as seen in normal rat hepatocytes.

It was seen that saccharin was more potent than phenethylbiguanide (phenformin) in its effect on the insulin-treated diabetic hepatocytes. This observation is in contrast to the effect of saccharin in normal and diabetic rat hepatocytes when the compounds containing the guanadino group are more effective than the cyclic sulphonamide group in saccharin.

When cells isolated from insulin-treated diabetic rats are exposed to diazoxide, the activity of glycogen phosphorylase a is decreased and the compound behaves as if it was an insulin-mimetic although diazoxide is known to be a hyperglycaemic drug. In

this respect, diazoxide gave a paradoxical effect but the mechanism of this effect is not understood. This effect of diazoxide is in contrast to the effect on the normal rat hepatocytes.

CONCLUSION

The effect of biguanides and guanamines on the glycogen phosphorylase a in insulin treated diabetic rat were much more like the effect of the compounds on the normal rat hepatocytes. Some differences did appear, however, such as the unexpected effects of the cyclic sulphonamides, saccharin and diazoxide, indicating that they may work by different mechanisms.

4. 4. Effect of biguanides, guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a from normal rats in the presence of different concentrations of glucose.

As has been discussed above, the effect of synthesised biguanides, guanamines and insulin depends on what animals the cells have been isolated from i.e. there are different effect in hepatocytes from control , diabetic and insulin-treated diabetic rat. The next series of experiment were performed to try and elucidate the reason for this difference in effect of the compounds on glycogen phosphorylase activity by preincubation of normal rat hepatocytes in different concentrations of glucose. There are similarities between the enzymatic change induced in the present study by different glucose concentrations in the isolated liver preparation and the findings of Hue *et al.* (1975) and Witter and Avruch (1978). In the liver of rats *in vivo* Stalmans *et al.*, (1974), indicated that the isolated preparation may be used with confidence in the investigation of the effect of biguanides, guanamines and insulin on the activity of glycogen phosphorylase in the presence of different concentrations of glucose. Since biguanides,

guanamines and insulin have been shown to have an influence on glycogen phosphorylase in different types of cells in normal, diabetic and insulin treated diabetic rat and have been implicated in the overall picture of the diabetic condition, it was considered pertinent for us to extend this investigation to the effect of biguanides, guanamines and insulin on rat hepatocytes in normoglycaemic (10mM glucose) and hyperglycaemic conditions (30mM glucose) to try and reproduce the metabolic conditions of the diabetic rat, inother words hyperglycaemia. It was noted that the effects of biguanides, guanamines and insulin on hepatic glycogen phosphorylase a in normal cells preincubated in 30mM glucose were very similar to those for cells isolated from diabetic animals. This result suggested that there is no difference between the cells themselves whether isolated from normal or diabetic rats but only that they have been exposed to different glucose concentrations in the animal. It was further demonstrated that an increase in the glucose concentration of the medium caused a rapid and extensive fall in the level of phosphorylase a in the liver. This effect is adequately accounted for by the stimulation of the phosphorylase phosphatase reaction by glucose (Stalmans *et al.*, 1970). This effect is said to be the result of binding of glucose to glycogen phosphorylase a (Stalmans *et al.*, 1974). Carabaza and co-workers (1992) confirmed that glucose is the molecule responsible for the inactivation of glycogen phosphorylase (Watson *et al.*, 1994) and at the same time they clearly demonstrated that, contrary to what Stalmans and co-worker (1974) observed, that the inactivation of glycogen phosphorylase is a signal for glycogen synthase activation. Carabaza and co-workers (1992) have used several glucose analogues such as deoxyglucose and 5-thioglucose, which caused the inactivation of glycogen phosphorylase and the activation of glycogen synthase. However, 6-deoxglucose and 1,5-anhydroglucitol inactivate phosphorylase without increasing the activation state of glycogen synthase, indicating that the two effects are not causally related.

Acute hyperglycaemia causes a marked inhibition of hepatic glucose production in normal rats mainly through the suppression of glycogenolysis (Rossetti *et al.*, 1993; Liu *et al.*, 1993). Increasing the concentration of glucose in the medium reduced and

even fully suppressed (20mM glucose) glycogen breakdown (Meury *et al.*, 1994). In our studies, both biguanides and guanamines mimic insulin in the inactivation of glycogen phosphorylase in the presence of 10mM glucose. When the concentration of glucose is increased in the medium to 30mM, the effect of biguanides and guanamines on glycogen phosphorylase was reversed and an increase in the activity was seen. This result is similar to that of Bollen *et al.* (1983), who showed that phosphorylase was partially inactivated in the presence of 5mM glucose and almost completely inactivated at a very high glucose concentration (60mM). With intermediate glucose concentrations, the same inactivation of phosphorylase was observed as seen in this study.

When hepatocytes in a high glucose medium (30mM) are exposed to biguanides, guanamines and insulin there is an increase in the activity of glycogen phosphorylase to a level which does not exceed the basal phosphorylase activity when hepatocytes are in 10mM glucose. From this observation we can speculate that biguanides, guanamines and insulin correct the effects of the hyperglycaemic state by restoring glycogen phosphorylase activity back to near the control level. It is interesting to note that when the activity is decreased by biguanides, guanamines and insulin in 10mM glucose and when the activity is increased by biguanides, guanamines and insulin in 30 mM glucose, the activity reached approximately the same level. It must be assumed that the increase in enzyme activity is to correct the defect which results from exposure of the cell to a high concentration of glucose (30mM). Weak inhibitors of glycogen phosphorylase are weakly hypoglycaemic (Kasvinsky *et al.*, 1978). Amylin, the major component of amyloid fibres in the pancreas of NIDDM patients, causes a dose-dependent increase in phosphorylase activity (Young *et al.*, 1991). Our observations and those of Martin *et al.*, 1991 suggest that inhibitors of glycogen phosphorylase may help shift the balance between glycogen synthesis and glycogen degradation in favour of glycogen synthesis in both muscle and liver. Therefore they may be useful therapeutic agents for the treatment of diabetes.

A clear structure-activity relationship was observed when cells were pre-incubated in 10 mM and 30 mM glucose. When hepatocytes, preincubated in 10mM glucose, were exposed to biguanides, the most active compound was benzylbiguanide. By changing the concentration of glucose to 30mM and using the same series of biguanides, it appeared that the most potent compound was still benzylbiguanide although the effect was different (the effect was always similar to insulin). This is in agreement with *in vivo* experiments which show that anti hyperglycaemic biguanide increase the blood glucose-lowering efficacy of insulin in hyperglycaemia (Pagono *et al.*, 1983; Gin *et al.*, 1985; Prager *et al.*, 1986; Leblanc *et al.*, 1987; Nosadini *et al.*, 1987; Hother-Nielsen *et al.*, 1989; Reaven *et al.*, 1992).

Cyclization of biguanides to the corresponding 1,3,5-triazine still gives compounds that mimic insulin. When control hepatocytes are incubated in 10mM glucose, benzylguanamine seemed to have more effect on activity than phenyl- or phenethylguanamine. When the concentration of glucose was increased to 30mM and the same series of guanamines was tested, there was little change in enzyme activity. but it was observed that the phenethylguanamine was more effective than phenyl- or benzylguanamine.

Benzylguanamine > phenylguanamine > phenethylguanamine (normal)
Phenethylguanamine > phenylguanamine > benzylguanamine (diabetic)

From these observations we can suggest that the cells isolated from diabetic rats differ only from normal cells in that they have been pre-exposed to high glucose concentrations *in vivo*.

In a comparison between phenethylbiguanide (phenformin) and 1,1-dimethylbiguanide (metformin), to determine the requirements for cyclisation of the biguanide for the possession of hypoglycaemic activity (the effect on glycogen phosphorylase) it was seen that cells were more sensitive to 1,1 dimethylbiguanide (metformin) than phenethylbiguanide (phenformin) in 10mM glucose but that there

was greater response to phenethylbiguanide than 1,1-dimethylbiguanide in 30mM glucose. Again the same was observed in diabetic hepatocytes as was observed in normal rat hepatocytes when preincubated in 30mM glucose which confirms the theory that the cells from the diabetic rat seem to be simply normal cells pre-exposed to high glucose concentrations. Experimental studies have demonstrated that the anti-hyperglycaemic effects of metformin in the clinical condition of NIDDM are primarily due to suppression of hepatic glucose production, which is mediated, at least in part, by suppression of free fatty acid and lipid oxidation (Venura *et al.*, 1994). Presumably, the biguanides also affect hepatic glucose production through decreasing the activity of glycogen phosphorylase in 10mM glucose and increasing the activity in 30mM glucose.

A number of structural features of biguanides were examined to further define the breadth of their activity. One examination involved comparison between phenethylbiguanide (phenformin) and o-sulfobenzimide (saccharin). The latter reduced the activity more than phenethylbiguanide when the cells were preincubated in 10mM glucose but when the concentration of glucose was increased to 30mM glucose, the phenethylbiguanide still increased the enzyme activity significantly at all concentrations whereas the effect of saccharin was only seen at high concentration. The same effects as found in 30mM glucose were also observed in diabetic hepatocytes.

CONCLUSION

1. The effect of biguanides and guanamines mimic insulin in decreasing glycogen phosphorylase a activity in the normal rat hepatocytes preincubated in 10mM glucose and increasing the activity in the same cells preincubated in 30mM glucose. The latter effect is similar to the effect in diabetic hepatocytes. From the above findings, it can be concluded that the hepatocytes isolated from the diabetic rat are similar to normal cells pre-exposed to high glucose concentrations.

2. The most active compound of biguanides series was benzylbiguanide in concentrations of 10mM and 30mM glucose. They were hence considered to be worthy of further development as substituted biguanides.

3. Cyclization of biguanides to the corresponding 1,3,5- triazine still gave compounds which inhibited the activity of glycogen phosphorylase in 10mM glucose and increasing the activity in 30mM glucose.

4. In the cyclised guanamine series, the most potent compound was phenethylguanamine in cells preincubated in 30mM glucose.

5. Cyclization is thought to be necessary for action in 30mM glucose since phenformin was more effective than metformin. This again is similar to the effects in diabetic hepatocytes.

6. Further structure - activity relationship studies demonstrated that the guanadino group is probably necessary for activity since saccharin did not behave as a hypoglycaemic drug in high glucose.

4. 5. Effect of biguanides, guanamines and insulin on the activity of glycogen phosphorylase a in hepatocytes isolated from STZ diabetic rats in different glucose concentration.

In comparing between the effects of the synthesised compounds in normal rat hepatocytes preincubated in 10mM and 30mM glucose (previous section) and diabetic hepatocytes (section 4.2), it was suggested that there is no difference between the cells isolated from normal and diabetic rats since the effect of the compounds in normal cells preincubated in 30mM glucose was the same as the effect in diabetic hepatocytes. In this section we have tried to see the effect of the same series of compounds on the hepatocytes isolated from STZ diabetic rats preincubation in 10mM and 30mM glucose to confirm this hypothesis.

The results show that inactivation of glycogen phosphorylase a by glucose is not impaired in diabetic hepatocytes, although basal phosphorylase a activity is lower than in normal rat hepatocytes. This result is in a agreement with Miller and co-workers (1973) who have reported that glycogen phosphorylase was inactivated by glucose in livers isolated from rats made diabetic for 2-6 days (Miller *et al.*, 1973; Miller, 1978 b) and the observation from Bollen *et al* (1983) on the effect of glucose on glycogen phosphorylase in hepatocytes from acutely (40h) and chronically (90h) alloxan - diabetic rats. Bollen found that the glucose- induced inactivation of phosphorylase proceeded normally in all conditions. Similar observation were made in a study on the response to glucose and fructose in streptozotocin-diabetic rats. When hepatocytes isolated from diabetic rats were incubated with 20mM glucose plus 3mM fructose this resulted in a more intense inactivation of phosphorylase than when 20mM glucose was added alone (Ciudad *et al.*, 1988). Inactivation of phosphorylase by glucose results from direct interaction of glucose with the enzyme, rendering it a better substrate for phosphorylase phosphatase (Stalmans et al, 1971; Hers, 1981).

It was observed from our results that all of the synthesised compounds mimic insulin in stimulating the activity of glycogen phosphorylase when the diabetic cells are preincubated in 10mM glucose and in decreasing glycogen phosphorylase a activity following preincubation of the cells in 30mM glucose. This is the exact opposite of the effects seen in control cells. In the biguanide series , the most active compound was phenylbiguanide when the diabetic cells were preincubated in either concentration of glucose (10 or 30mM). In the cyclised guanamine series the most potent was phenethylguanamine in 10mM and 30mM glucose which confirmed that the stabilisation of phenethylbiguanide by cyclization to a triazine might results in enhanced anti-diabetic potency. It was observed that the effect of biguanides and guanamines on the diabetic cells incubated in 30mM glucose was opposite to that seen in hepatocytes from normal rats. From this result we can suggest that the cells isolated from the diabetic rat are **not** normal since the effect of the compounds on hepatocytes isolated from STZ diabetic rats was **opposite** to that seen in normal rat

hepatocytes. This results also suggests that guanamines act as insulin-mimetics in potentiating the effect of glucose on the glycogen phosphorylase in diabetic cells. The reasons for this are, however, unclear.

CONCLUSION

1. All of the synthesised compounds mimic insulin by stimulating the activity of glycogen phosphorylase a when the diabetic cells were preincubated in 10mM glucose and decreasing glycogen phosphorylase a activity in cells preincubated in a glucose concentration of 30mM.

2. It was observed that the effect of biguanides and guanamines on diabetic cells incubated in 30mM glucose is opposite to that seen in hepatocytes from normal rats hepatocytes. This result suggests that the cells isolated from STZ diabetic are not the same as normal cells.

3. In the biguanide series, the most active compound was phenylbiguanide when the diabetic cells were preincubated in either 10mM or 30mM glucose.

4. In the cyclised guanamine series the most potent was phenethylguanamine in both 10mM and 30mM.

4. 6. ANDROST-4-ENE-3,17-DIONE METABOLISM IN ISOLATED NORMAL AND STREPTOZOTOCIN DIABETIC RAT HEPATOCYTES.

Previous studies using liver microsomes, have indicated that diabetes mellitus can affect steroid metabolism in rats and this effect can be reversed by insulin administration to diabetic rats (Skett, 1986). Reports from various studies have indicated that streptozotocin (STZ)- induced diabetes mellitus can also influence

hepatic steroid metabolism in rats (Leaming *et al.*, 1982; Subbiah & Yunker, 1984). The effect of diabetes on hepatic steroid metabolism can be prevented or reversed by insulin administration to diabetic animals. Diabetes, produced by streptozotocin, can induce CYP4A2 and P450K-2 (similar to CYP2C23) in rat kidney. Treatment of diabetic rats with insulin reversed the increase in the levels of CYP4A2 and P450K-2 (Imaoka *et al.*, 1993)

In this study it has been shown that STZ- induced diabetes mellitus causes a change in the metabolism of androst-4-ene-3,17-dione. The change in the enzyme activities were dependent on the enzyme being studied, with the basal activity of the cytochrome P450-dependent enzymes (7α-, 6β- and 16α-hydroxylases) being increased while the cytochrome P450-independent enzyme ,17-OHSD, was decreased and 5α- reductase was unaffected in diabetes. This data is in partial agreement with the results obtained from studies using hepatic microsomes (Reinke et al., 1978; Skett, 1986) in the elevation of 7α- and 16α-hydroxylase and no effect on 5α- reductase and also in agreement with that of Hussin and Skett (1988) who observed, using 3 day diabetic hepatocytes, an increase in 7α-hydroxylase and decrease in 17-OHSD and with the results of Schenkman (1991) using diabetic male rat hepatocytes in the elevation of 7α-hydroxylase. Hussin (1988) observed that the effect of diabetes mellitus on steroid metabolism in the rat is time-dependent and is not the same in the acute and chronic phase. As these experiments performed by Hussin were carried out *in vitro*, differences between these results and those reported here may be due to different experimental conditions, the number of days following induction of diabetes and the effect of extra hepatic factors. Insulin in normal rat hepatocytes acts as a general stimulator of the enzymes in the liver metabolising androst-4-ene-3,17-dione.

Phenethylbiguanide and phenethylguanamine were tested in this study in assays for insulin-like activity using the insulin-sensitive metabolism of steroids by isolated rat liver cells (Hussin, 1988).

CONCLUSION

From the above finding it can be concluded that STZ- induced diabetes mellitus causes a change in the metabolism of androst- 4- ene- 3,17- dione. The changes in the enzyme activities were dependent on the enzyme being studied, with the basal activity of cytochrome P450-dependent enzymes (7α-, 6β- and 16α-hydroxylases) being increased while the cytochrome P450-independent 17-OHSD was decreased and 5α-reductase was unaffected in diabetes.

4.7. THE EFFECT OF PHENETHYLBIGUANIDE, PHENETHYLGUANAMINE AND INSULIN ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN NORMAL RAT HEPATOCYTES.

The insulin-mimetic effects of phenethylbiguanide and phenethylguanamine in increasing androst- 4- ene- 3,17- dione were examined, in order to extend our knowledge of the insulin-like actions of biguanides and guanamines from glycogen metabolism to steroid metabolism. As shown in this study, phenethylbiguanide increased the activity of the cytochrome P450-dependent enzymes (7α-, 6β- and 16α-hydroxylases) at low concentration (10^{-9}M to 10^{-6}M). The maximum effect was observed only at 10^{-9}M and 10^{-7}M. Investigations from our laboratory indicated that phenethylbiguanide has a direct effect on the liver in elevating androst-4-ene-3,17-dione metabolism. It is able to mimic the effect of insulin in normal and diabetic rat hepatocytes with respect to androst-4-ene-3,17-dione metabolism (Hussin, 1988). Vandate (another oral hypoglycaemic) has been shown to have an insulin- like effect

on hepatic glycogen metabolism in non diabetic and streptozotocin - induced diabetic rats (Pugazhenthi & Khandelwal., 1990) and appears to exert insulin- mimetic actions on the P4503 and P4501 family of proteins (Verrecchia & Guaitani, 1993). Insulin also has this effect (Hussin & Skett, 1988).

Phenethylbiguanide exerted an inducing effect on the cytochrome P450 content in isolated hepatocytes from rats with experimental diabetes and this was markedly greater than observed in normal hepatocytes. When phenethylbiguanide was added to isolated hepatocytes from normal rats, induction of cytochrome P450 was observed only in the presence of added dibutyryl cAMP (Canepa *et al.*, 1990). A study demonstrated that phenethylbiguanide stimulated a high affinity of isolated liver plasma membranes to cyclic AMP phosphodiesterase in a dose-dependent manner, decreasing the intercellular cyclic AMP content of isolated hepatocytes without being effective on plasma membrane- bound adenylate cyclase (Luly *et al.*, 1977). The stimulation of liver membrane bound cyclic AMP phosphodiesterase by phenethylbiguanide led to a comparison of this effect with the similar effect known to be exerted by insulin on liver membranes (Tria *et al.*, 1977).

Elevation of cyclic AMP levels, either directly or indirectly, has been shown to produce an inhibitory effect upon the metabolism of the steroid substrate, androst-4-ene-3,17-dione (Berry & Skett 1988). A role for cyclic AMP in the regulation of monooxygenase activity has been provided by Weiner and co- workers (1972), where it was reported that, *in vivo*, dibutyryl-cyclic AMP can inhibit metabolism of hexobarbitone. From these observation it appears that cAMP can inhibit cytochrome P450 whilst phenethylbiguanide activates cytochrome P450. It is possible to speculate that phenethylbiguanide has it effect on cytochrome P450 by inhibiting the generation or action of cAMP.

Similarly, phenethylbiguanide increased the activity of 17-OHSD significantly with maximum effect observed at low concentration (10^{-9}M). The effect at the therapeutic concentration of 10^{-5}M (157% of the control) was similar to that seen by Hussin and Skett (1988). At a concentration of 10^{-8}M, phenethylbiguanide gave the

maximum response on the activity of 5α-reductase in this study. This is in contrast to the study of Hussin (1988) where there was no significant change at a concentration of 10^{-5}M in the control.

Several observations on the mechanism of action of the biguanide group suggest that these compounds have an effect at the post-receptor level. Metformin, in rat hepatomas cells, enhanced both basal and insulin-stimulated glucose incorporation into glycogen in a time- and dose-dependent manner and had no effect on insulin binding to the receptor. These studies suggest, therefore, that metformin may influence cellular metabolism by potentiating insulin action through a mechanism that goes beyond insulin receptor binding (Gullo, 1988). Biguanides have also been reported to have a role in the alteration of insulin stimulation of glycogen synthesis without affecting the insulin receptor binding (Alengrin *et al.*, 1987). Metformin (1,1 dimethylbiguanide) potentiates the acute action of insulin at the post-receptor level in normal liver cells by increasing the insulin- dependent stimulation of glycogen and lipid synthesis without alterating the insulin binding receptor (Melin *et al.*, 1990). These observations suggest that biguanides exert their effects on androst-4-ene-3,17- dione metabolism by acting through mechanisms that go beyond insulin receptor binding in a way which has yet to be determined.

The cyclization of phenethylbiguanide to phenethylguanamine improved the effect of the compound on the activity of cytochrome P450-dependent enzymes (7α-, 6 β-, 16α-hydroxylases). Hepatocytes become more responsive to phenethylguanamine with the activity the 7α-, 6β- and 16α-hydroxylases increasing to 712%, 750% and 730% of the control respectively. This effect appeared to be two to three times greater than phenethylbiguanide. Phenethylguanamine showed a decrease in the activity of 17-OHSD in contrast to the effect of phenethylbiguanide. There was no improvement in the effect on the activity of 5α-reductase when phenethylbiguanide was cyclised to phenethylguanamine. It is interesting to note that only the cytochrome P450-dependent enzymes were involved. This possibly indicates that the three cytochrome

P450-dependent enzymes are more susceptible to alteration by a triazine ring than by a biguanide but that the flavin-dependent enzymes are not.

When the same cells were exposed to insulin, there was a significant increase in all of the enzyme activities and the maximum effect was observed at the physiological concentration of 10^{-9}M. This is in agreement with the result of Hussin and Skett (1988) in his study using the same incubation time. The role of insulin in the preservation and induction of cytochrome P450 isoenzyme activities and contents was investigated in rat hepatocytes for 4 days (Saad *et al.*, 1994). The results of these investigations demonstrate that different tissue oxygen tension modulates the responsiveness of the cultured hepatocytes to insulin and glucagon. This modulation results in an altered activity of cytochrome P450 isoforms. Pyerin *et al* (1983) have reported the phosphorylation of monooxygenase components caused conversion of cytochrome P450 to its denatured form, cytochrome P420 (Taniguchi *et al.*, 1985) and is dependent on the presence of both ATP and cAMP-dependent protein kinase suggesting that cytochrome P450 is a physiological substrate of cAMP-dependent protein kinase. Pyerin *et al* (1984) also reported a significant decrease in monooxygenase activity when cytochrome P450 was phosphorylated. Thus one of the mechanisms which could elicit an increase in 7α-, 6β- and 16α-hydroxylase is decreasing the intracellular concentration of cAMP.

CONCLUSION

Phenethylbiguanide and phenethylguanamine are both able to mimic the effect of insulin in normal rat hepatocytes with respect to androst-4-ene-3,17-dione metabolism. However the cyclization of phenethylbiguanide to phenethylguanamine gives a compound which is only effective on the cytochrome P450-dependent enzymes (7α-, 6 β- and 16α-hydroxylases). This possibly indicates that the three cytochrome P450 dependent enzymes are more susceptible than the flavin-dependent enzymes to the triazine ring in phenethylguanamine.

4.8 GENERAL DISCUSSION

All of the results can be summarised as follows:

1. The effect of biguanides, guanamines, miscellaneous compounds and insulin on the activity of glycogen phosphorylase a in rat hepatocytes

i) *In normal rat hepatocytes*: the effect of biguanides and guanamines on the glycogen phosphorylase a is an insulin-like effect in inactivating the enzyme. A clear structure - activity relationship was observed and the most active compounds of the biguanides was benzylbiguanide. When the biguanides were cyclised to the corresponding 1,3,5-triazine (guanamine) the compounds still inhibited the activity of the enzyme, the most active compound in this series was benzylguanamine .

Cyclization is, however, not necessary for activity on the normal rat hepatocytes. 3,5-diamino-1,2,4-triazol exhibited glycogen phosphorylase lowering activity similar to the biguanides and guanamine which suggested future work on cyclization of biguanides to a triazole ring instead of a triazine. Cyclic sulphonamides e.g. in saccharin also had the same effect on glycogen phosphorylase activity as the biguanides, perhaps due to similarities in structure elucidated by molecular graphics techniques.

ii) *In diabetic rat hepatocytes:* The effect of biguanides and guanamines still mimic insulin in that they increase glycogen phosphorylase a activity. However the rank order of potency was opposite to that seen in hepatocytes from control animals. It was indicated that the cyclization of biguanides may be necessary for action in diabetic hepatocytes. Studies on 3,5-diamino-1,2,4-triazole demonstrated that the presence of a triazine ring may be important for the activity.

iii) *In insulin treated diabetic rat hepatocytes:* These comparative experiments indicated in general that cells isolated from insulin-treated diabetic rats responded in a similar fashion to those isolated from control animals.

iv) *In normal rat hepatocytes in the presence of different concentrations of glucose:* The series of experiment were performed to try to elucidate the reason for the difference in effect of compounds on glycogen phosphorylase activity in different types of cells. A similar structure-activity relationship was observed in control cells preincubated in 30mM glucose as in cells isolated from STZ-diabetic rats, indicating that the latter cells are, perhaps, simply control cells that have been pre-exposed to high glucose concentrations.

v) *In diabetic rat hepatocytes in the presence of different concentrations of glucose*: It was observed from these results that all of the synthesised compounds mimic insulin in stimulating the activity of glycogen phosphorylase when the diabetic cells are preincubated in 10mM glucose and by decreasing glycogen phosphorylase a activity in cells preincubated in 30mM glucose. This result suggests that cells isolated from STZ-diabetic rats are **not** normal and respond differently to cells isolated from control liver.

2. The effect of phenethylbiguanide phenethylguanamine and insulin on androst -4-ene-3,17-dione metabolism in normal rat hepatocytes.

The insulin-mimetic effects of phenethylbiguanide and phenethylguanamine in increasing androst -4- ene- 3,17- dione metabolism were examined in order to extend our knowledge of the insulin-like action of biguanides and guanamines from glycogen metabolism to steroid metabolism. Phenethylbiguanide increased the activity of the cytochrome P450-dependent enzymes. Similarly phenethylbiguanide increased the activity of 17-OHSD significantly with maximum effect observed at the low concentration of 10^{-9}M.

The cyclization of phenethylbiguanide to phenethylguanamine improved the activity on the cytochrome P450-dependent enzymes. This effect appeared to be 2-3 times greater than with phenethylbiguanide. Phenethylguanamine gave a decrease in the activity of 17-OHSD in contrast to the effect of phenethylbiguanide. It is interesting to note that only the cytochrome P450-dependent enzymes were positively affected by the guanamine. This possibly indicates that the three cytochrome P450-dependent enzymes are more susceptible to alteration by the triazine ring.

Overall, biguanides and guanamines have been shown to have significant, direct, insulin-like effects on glycogen phosphorylase a activity and androst-4-ene-3,17-dione metabolism in hepatocytes isolated from control and STZ-diabetic rats. The effectiveness of the compounds depended on the enzyme under study and on the original state of the animal (diabetic or not).

Further investigation is warranted on the usefulness of congeners of the presently used biguanide, phenformin, and on the cyclised biguanides, such as the guanamines and, perhaps, the five-membered structures, the triazoles.

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ABBREVIATIONS

[Ca2+]i	Intracellular free calcium ion concentration
DTT	DL-Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
Glc1p	Glucose-1-phosphate
HEPES	N-[2-hydroxyethyl]piperazine- N'[2 ethanesulfonic acid]
MOPS	3-[N-Morpholino]propanesulphonic acid
STZ	Streptozotocin
T.L.C	Thin layer chromatography
AMP	Adenosine-5'-monophosphate
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5-triphosphate
D.P.M	Disintegrations per minute
CyclicAMP	Cyclic adenosine 3',5'- monophosphate
g	Centrifugal g-force
I.U	International unit
EGTA	Ethylene glycol-bis (β- aminoethyl ether). N,N,N,N-tetra-acetic acid
OHSD	Oxosteroid oxidoreductase
Km	substrate concentration producing half-maximal volecity.
G-6-P	Glucose-6- phosphate.
NIDDM	Non- insulin- dependent diabetes mellitus.
S.D	Standard deviation
Ser	Serine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
kDa	Kilodalton

Publications.

Several aspects of the work described in this thesis have been published.

1. Al-Shibani, N; Skett, P. (1994). Synthesis of guanamines and their effect on glycogen phosphorylase a activity. Br. J. Pharmac. 113, 96P.

2. Al-Shibani, N; Skett, P. (1994). Effect of guanamines on glycogen phosphorylase in cultured hepatocytes from streptozotocin diabetic rats. Br. J. Pharmac. 113, 97P.

3. Al-Shibani, N; Skett, P. (1995). Effect of guanamines (cyclised biguanides) on glycogen phosphorylase a activity in hepatocytes from normal rats in different glucose concentrations. Br. J. Pharmac. Abstract (accepted).

4. Al-Shibani, N; Skett, P. (1995). A change in the effect of guanamines on glycogen phosphorylase a activity in hepatocytes from streptozotocin diabetic rats in different glucose concentrations. Br. J. Pharmac. Abstract (accepted).

5. Al-Shibani, N; Kirby, G.W; Skett, P. (1995). Synthesis of biguanides and guanamines and their effect on glycogen phosphorylase a in cultured hepatocytes from normal and streptozotocin induced diabetic rats. Br. J. Pharmac. (submitted).